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An in vitro evaluation of tyrosine derived polyarylates and polycarbonate as polymeric scaffold for human mesenchymal stem cells osteogenic differentiation

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ABSTRACT

AN IN *VITRO* EVALUATION OF TYROSINE DERIVED POLYARYLATES AND POLYCARBONATE AS POLYMERIC SCAFFOLD FOR HUMAN MESENCHYMAL STEM CELLS OSTEOGENIC DIFFERENTIATION

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Human mesenchymal stem cells (hMSCs) obtained from the adult bone marrow are multipotential cells that are capable of differentiating along several lineages. They are readily available and abundant source of cells in the field of tissue engineering. For their use in treating certain connective tissue defect or disorders, their success depends at minimum on the use of scaffolds that support differentiation. Therefore , this thesis details a study that systematically evaluated hMSC differentiation on materials that exhibit gradual range in surface properties as a first study for selecting materials as potential scaffolds.Polycarbonates and polyarylates have combinatorial libraries that exhibit a range of surface chemistries leading to changes in the surface properties such as wettability, rigidity and protein adsorption. Structural modifications of their pendant chain and backbone provide a means to modify their physicomechanical, chemical and biological properties. Polymers were coated onto 96-well cell culture plates using the solvent casting technique. The human MSCs isolated from the bone marrow were seeded at a fixed density of 3×10^4 cells/cm^2 in 96-well plates containing coated polymers. The cells were grown for 16 days in the presence or absence of osteogenic supplement. Alkaline phosphatase activity, cell number by DNA quantification and the amount of calcium in the extracellular matrix were measured at day 4,8,12 and 16. In addition, osteocalcin assay was performed at day 16. The results indicated that cell proliferation

was highest on more hydrophilic surface; however the osteogenic differentiation was greater on more hydrophobic polymeric substratum.

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AN IN *VITRO* EVALUATION OF TYROSINE DERIVED POLYARYLATES AND POLYCARBONATE AS POLYMERIC SCAFFOLD FOR HUMAN MESENCHYMAL STEM CELLS OSTEOGENIC DIFFERENTIATION

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This thesis is dedicated to my parents and my younger sister “Shacha”.

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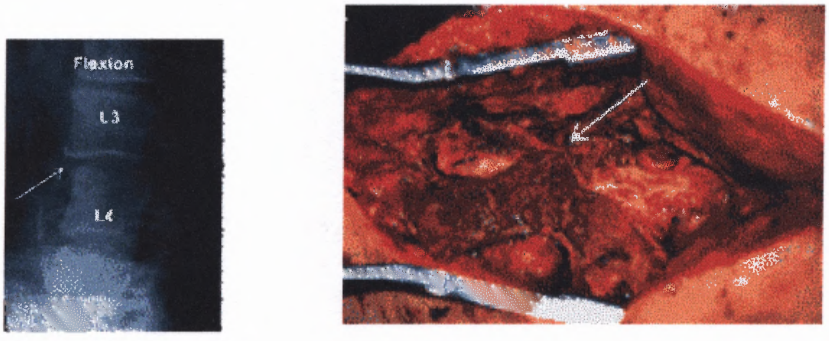
CHAPTER 1

BACKGROUND

Tissue Engineering is a multidisciplinary field that applies the principles of biology and engineering to develop tissue substitutes to restore, maintain, or improve the function of diseased or damaged human tissues. It is principally focused on restoring the functionality of an organ, rather than just its anatomy.

Every year, millions of surgical procedures are performed that require tissue or organ substitutes to repair or replace damaged or diseased organs or tissues. There are currently on the order of 500,000 bone grafts performed annually in the United States [54]. For example, there were approximately 220,000 spinal fusion cases performed in 1998 requiring bone grafts to secure areas of the spine affected deformity, trauma, tumors, or degenerative disc disease as depicted in the figure 1.1. Also, there are 170,000 fractures in the United States that fail to heal each year and are diagnosed as non-union [54]. Figure 1.1 shows an example of a non-union of the femur.



(a) (b)

Figure 1.1 Example cases requiring bone substitutes[54].

Conventional approaches to address the need for bone substitutes include:

1. **Autografting** involves harvesting a bone from one location in the patient's body and transplanting it into another part of the same patient. Using autologous grafts, when available, typically produces the best clinical results. Autografting is considered the gold standard. An example of one the most commonly performed bone autografting procedures is for use in spinal fusion. In a fusion procedure, bone graft from the patient's hip is implanted in disc spaces between spinal vertebrae or along the back of the spine (Figure 1.2). The grafted bone fuses the vertebrae together over several months. Use of autogenous tissue avoids the issues of immunogenicity. Autografting, however, has several associated problems including the additional surgical costs for the harvesting procedure, and infection and pain at the harvesting site. For example, harvesting an ileac crest graft (i.e., the protruding bony section of the patient's hip) can cost between $1000 to $9,000/procedure for the harvesting operation and the additional hospital stay (2). The morbidity at the harvest site can be tremendous with problems such as pain, infection, and blood loss requiring blood transfusion adding the associated risks of transfusion reaction and blood borne infection.

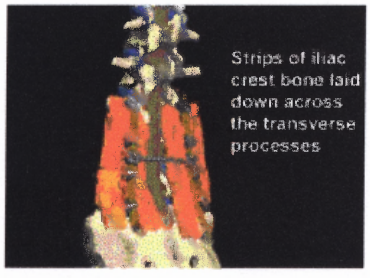


Figure 1.2 Autografting procedure to repair collapsed disc [54].

2. Allografting involves harvesting tissue or organs from a donor and then transplanting it to the patient. The donor might have recently died and donated heart, kidney, liver, bone, pancreas, etc. Living donors might also be used to donate lungs and kidneys. While transplantation technology has dramatically improved over the past several decades with the advent of anti-rejection drugs, the immediate problem is shortages in donor availability. For example, in 1998, there were 22,170 organ transplants in the U.S., but 4,855 patients died while waiting for suitable donor organs Allogenic implants are acellular and are less successful than autografts for reasons attributed to immuogenicity and the absence of viable cells that become osteoblasts. Another disadvantage of allografting is concern with transmitted disease

3. Man-made materials, including metals, plastics, and ceramics represent approximately 8% of bone substitutes. These materials, however, are subject to fatigue, fracture, toxicity, and wear, and do not remodel with time (i.e., a metal bone implant cannot grow with the patient and it cannot change shape in response to the loads placed upon the implant. For all these reasons, there is a real need for alternative, off-the-shelf, bone substitutes and better wound healing therapies. Bone tissue engineering seeks to address this need.

While all these therapies have had significant medical impact, there are newer technologies on the horizon which seek to overcome the limitations of these 'conventional' approaches. These newer approaches include harvesting tissues and organs from cloned transgenic animals and gene therapies. While cloning and gene therapy have received much publicity, there are still significant technical, economical, political and ethical issues, which must be overcome before they become clinically available.

There are many approaches for bone tissue engineering. One approach for engineering bone tissue involves seeding highly porous biodegradable scaffolds with donor cells and/or growth factors, then culturing and implanting the scaffolds to induce and direct the growth of new, healthy bone tissue. Examples of other tissue engineered substitutes that are currently being investigated throughout the world include skin, cartilage, vasculature, heart, breast, and liver.

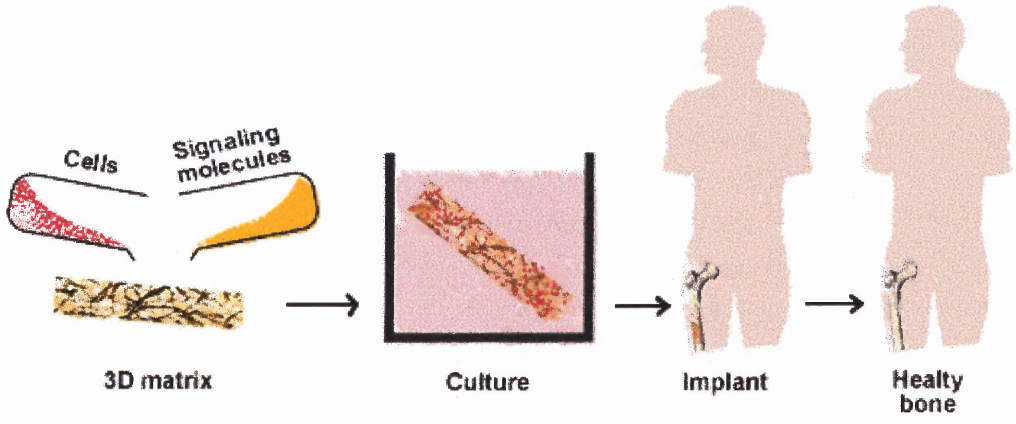


Figure 1.3 Schematic of a simple approach used in tissue engineering [54].

Conventional cell culturing involves growing cells in an artificial environment where they can thrive and replicate to form larger colonies of cells for applications such as diagnostic testing. These colonies, however, do not become organized into tissues or organs that could then be implanted back into the patient. Cell colonies need external cues or signals to grow into functional 3D tissues or organs. In the body, cells are constantly bombarded with mechanical, electrical, structural, and chemical signals hat trigger a desired cellular response. If these signals are not properly received or processed due to disease or trauma, then the cells dedifferentiate (i.e., become non-specific cell types), become disorganized, and eventually die.

The structural cues involve the interaction of cells with their extracellular matrix (ECM). The ECM is that part of our body which gives it form and shape. For example, bone is made up of an ECM composed of a composite fibrous network of collagen encased within a hard matrix of calcium/phosphorous. Bone cells (osteoblasts, osteoclasts, osteocytes) exist in a symbiotic relationship with the ECM, first creating it, then remodeling it, and in turn being regulated by it. The physical communication between cells and ECM directly and indirectly impacts cell shape and function, and these signals are all necessary for normal cellular activity.

Cell actions and their responses to various environmental signals, including mechanical, electrical, structural and chemical, are mediated by protein based molecules loosely referred to as growth factors. The cellular regulatory actions of growth factors in bone include migration of cells from one site to another, morphogenesis from one cell type to another, and mitogenesis or cellular proliferation. Growth factors are produced both locally by bone cells and systemically from other sites. In mediating extracellular communications, growth factors act directly on the very bone cell that produced them, act on neighboring cells surrounding the growth factor producing cell relay a single growth factor communication signal received by one cell to neighboring cells due to direct cell to cell interaction (juxtacrine effect), and act on cells distant from the site of growth factor production by traveling through the blood stream (endocrine effect). Within the local bone environment growth factors reside in the interstitial fluid, on the cell surface, and in the ECM. These growth factors are not only important for growth, development, and day-to-day maintenance of bone tissues, but are mobilized during times of bone remodeling and injury.

Understanding the role of the ECM, growth factors and their basic concepts can be utilized in developing 3D cell culturing techniques as the next technological level in the development of substitute tissues and organs. These techniques involve placing cells and/or growth factors in synthetic scaffolds (which acts as temporary ECMs) in order to help encourage the cells to organize into tissues or perhaps whole organs. Scaffold-guided tissue generation is one enabling technology for the emerging field of Tissue Engineering.

Another approach will be "ex vivo gene therapy" consisting of isolation of relevant determined stem cells or committed progenitors from mature adults or from animals, expansion of them ex vivo, transfection of them and selection of transfected cells ex vivo, and then reintroduction of the cells in vivo. Genetic engineering, however, has numerous hurdles to overcome to make this approach reliable, practical, safe, and generally accepted.

Instead of administering growth factors directly, it is also possible to use genes that encode those molecules. The genes are part of a plasmid, a circular piece of DNA constructed for this purpose. The surrounding cells take up the DNA and treat it as their own. They turn into tiny factories, churning out the factors coded for by the plasmid. Because the inserted DNA is free-floating, rather than incorporated into the cells' own DNA, it eventually degrades and the product ceases to be synthesized.

This thesis is focused on developing scaffolds for stem cells in bone tissue engineering. The following chapter introduces the basics of stem cells and the synthetic scaffolds which are used in the field of tissue engineering.

CHAPTER 2

STEM CELLS AND POLYMERS USED IN MEDICINE

This chapter provides an introduction to stem cells and their different types. It will also introduce us to basic polymers used in biological systems and provide information about the general characteristics of the polymers used in the study.

2.1 An Introduction to Stem Cells

Stem cells are a special kind of cells with a unique capability to renew itself for indefinite period, throughout the life of the organism. Under right conditions, or right signals, stem cells can give rise to many cell types that make up an organism. Stem cells originate from the fetus, embryos and adults and give rise to specialized cells. Broadly two types of stem cells are found in an organism- embryonic stem cells and adult stem cells.

2.1.1 Embryonic Stem Cells (ES)

ES cells are derived from the inner cell mass of the blastocyst. Studies of the ES cells derived from the mouse blastocyst became possible 20 years ago with the discovery of techniques that allowed the cells to grow in the laboratory [1]. These cells are pluripotent- that is the ability to give rise to differentiated cell types that are derived from all three germ layers of the embryo, endoderm, mesoderm and ectoderm. ES cells have been shown to differentiate into a variety of cell types. For example, mouse ES cells can be directed in *vitro* to yield vascular structures [2], neurons that release dopamine and serotin [3] and endocrine pancreatic islet cells [4].All three studies demonstrate that the

resulting differentiated cells function the same way as they would in *vivo*. Scientists were able to direct these cells toward various cell types. Table 2.1 provides a summary of the differentiating capability of these cells.

Table 2.1 Reported differentiated cell types from mouse embryonic stem cells in vitro[5].

Cell type	Reference
Adipocyte	6
Astrocyte	7
Cardiomyocyte	8,9
Definitive hematopoeitic	11.12,13
Dendritic cell	14
Endonthelial cell	15,2
Keratinocyte	16,2
Lymphoid precursor	17
Mast cell	18
Neuron	19,20
Oligodendrocyte	21,22
Osteoblast	23
Pancreatic islets	4
Primitive hematopoetic	8,11
Smooth muscle	2
Striated muscle	24
Yolk sac endoderm	8
Yolk sac mesoderm	8
Chondromyocyte	10

Figure 2.1 depicts ES cells in context of mouse development. Upper panel shows development of mouse embryo with the inner cell mass from which ES cells can be derived, highlighted in yellow (adapted from Cold Spring Harbor Laboratory Manual: Manipulating the Mouse Embryo). Lower panel shows lineage diagram of mouse development with lineages colonized by ES cells highlighted in blue. ES cells can produce hypoblast derivatives in vitro but rarely do so in vivo.

Research has also been carried out with human embryonic stem cells. Human embryonic stem cells were first derived from human blastocyst by group at University of

Wisconsin, Madison [25]. Their multilineage potential was well demonstrated by Odonrico et.al. [26]. However, few studies have been carried out with these cells, primarily because of ethical issue and because it is difficult to grow these cells *in vitro*.

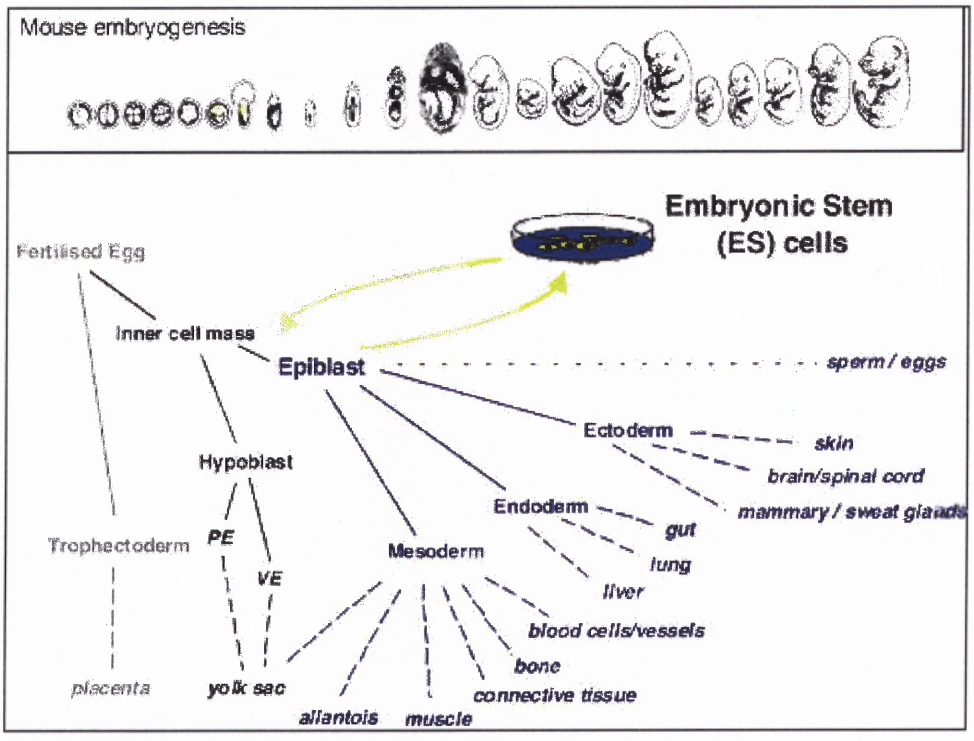


Figure 2.1 Diagram depicting ES Cells in context of Mouse Development [5].

2.1.2 Adult Stem Cells

Unlike ES cells, which are defined by their origin, there are no such defined characteristics for adult stem cells. The origin of adult stem cells is still not defined. Adult stem cells are rare. Their primary function is to maintain the steady state functioning of a cell and with limitations to replace cells that die because of injury or disease [27, 28]. They are found in many tissues. Adult stem cells from one tissue can generate differentiated cell types of other tissues, a property termed as "plasticity". Most of the information about adult stem cells comes from studies of mice. Previous experiments report that adult stem cells may assume the characteristics of cells that have

developed from the same primary germ layer or different germ layer.(refer figure 2.2). For example, many plasticity experiments involve stem cells derived from bone marrow, which is a mesodermal derivative. The bone marrow stem cells may then differentiate into mesodermally derived tissues such as skeletal muscle [29, 30], cardiac muscle [31, 32] or liver [33, 34, 35]. These cells may also differentiate into tissues that arise from different germ layer. For example, bone marrow-derived cells may differentiate into neural tissue, which is derived from embryonic ectoderm [36, 37]. And reciprocally, neural stem cell lines cultured from adult brain tissue may differentiate to form hematopoetic cells [38].

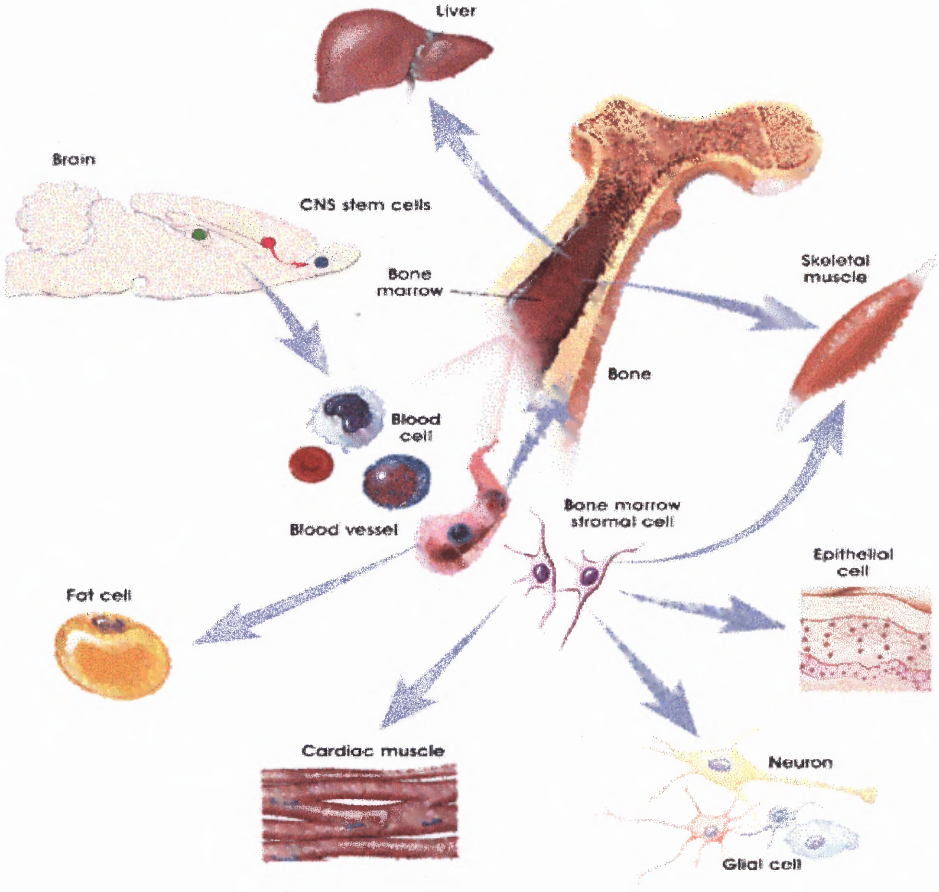


Figure 2.2 Evidence of Plasticity among Nonhuman Adult Stem Cells [39].

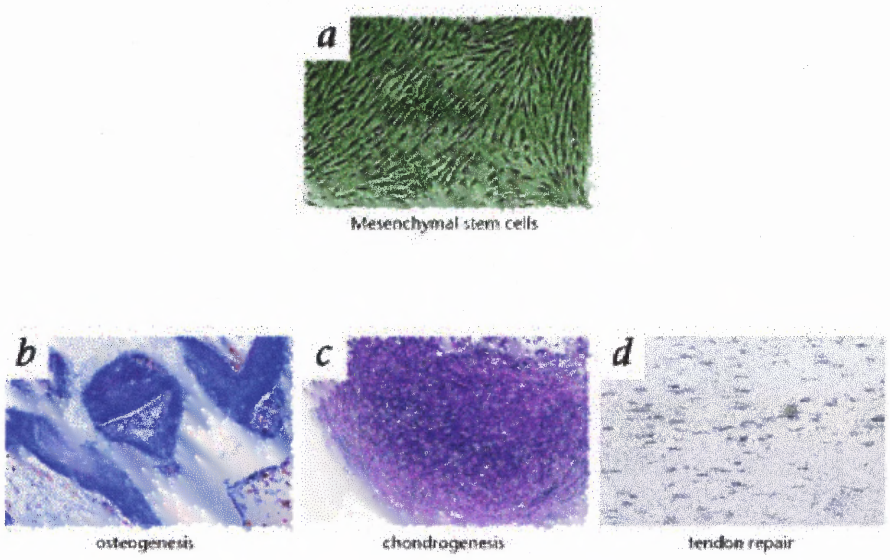


Figure 2.3 Differentiation Of Human Mesenchymal Stem Cells.
(a) Undiffferentiated MSC grown in culture, (b) Human MSCs coated onto ceramic cubes and placed in SCID mice differentiate into osteoblasts and osteocytes, which form bone. (c)Human MSCs, grown from a cell pellet after centrifugation, differentiate into cartilage. (d) Rabbit MSCs placed into a ruptured Achilles tendon sheath form tendon [40].

Thus there are many evidences that prove the plasticity of adult stem cells and embryonic stem cells. However the major difference between the two stem cell types that embryonic stem can proliferate indefinitely in culture, but there are no such reported experiments for adult stem cells. However, there are many reported studies for human adult stem cells as compared to human embryonic stem, primarily because of ethical issue. Moreover adult stem cells derived from human bone marrow have been studied extensively *in vivo* and *in vitro*. Also mesenchymal stem cells derived from bone marrow are easy to grow *in vitro* and are readily available. In this study the author is focused on the osteogenic differentiating potential of the human mesenchymal stem cells derived from bone marrow on different polymeric scaffolds. Scaffolds are one of the important factors in cell culture. In the following section the author has attempted to discuss various scaffolds used in tissue engineering with a prime focus on bone tissue engineering

2.2 Biopolymers

The success of tissue engineering depends on the ability of the cells to multiply, migrate and express normal physiological behavior in order to yield a cellular organization that performs the functions of the desired tissue. For example the engineering of living bone to repair skeletal defects has focused on growing osteoblasts on degradable polymeric [42, 43, 44, 45] and ceramic [46, 47, 48] matrices in vitro as well as in *vivo*. The matrix serves as the scaffold for bone cell proliferation and maturation. The cells form a bonelike tissue, that after implantation is fully integrated in to the patients own bone, thus repairing the bone injury or defect. Soon thereafter the scaffold resorbs away. Thus it is apparent that the scaffold material does play a vital role in tissue engineering. It is the interaction between the cell and the material that dictates whether the cells will proliferate, mature and express the desired tissue characteristics. Thus it is very important that the biomaterial in use should elicit desired cell response and should be biocompatible and biodegradable. Since biodegradable materials slowly degrade in the body, the need for a second surgery is eliminated. Further it reduces the health care cost and patient morbidity.

There are several biodegradable polymers available for biomedical applications, however, polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers (PLGA) remain the most popular and widely used for orthopedic applications. These FDA-approved biodegradable have been successfully used in sutures and in a number of drug-delivery applications. These materials are also examined for uses in tissue engineering applications such as bone, skin and liver tissues. These polymers are broken down in the body hydrolytically to produce polylactic acid and polyglycolic acid. Other

biodegradable polymers currently being used in tissue engineering include polycaprolactone, polyanhydrides, and polyphosphogenes. Several studies have examined the use of PGA based pins to treat pediatric fractures. For example, Svenson et al. [49] used 1.5-2.0 mm PGA pins to repair osteochondral and transphyseal fractures in children. Biodegradable screws have also been extensively used in foot surgery. In a large, 14 month study on the fixation of chevron osteonomies of the first metatarsal. 78 osteonomies were fixed with self-reinforced 2mm diameter PGA pins [50].Some inflammatory discharge of PGA material and fluid was found approximately 6 weeks postoperatively in two patients. In the past, biodegradable devices fabricated from PGA have exhibited various degrees of tissue reaction. For instance, in a study involving 40 patients, biodegradable PGA pins were used to treat displaced fractures of the distal wrist [51]. However inflammation was detected at the implantation site up to various time periods postoperatively (as long as 145 days in some cases), and a significant percent (22.5 %) of the patients required debridement of the inflamed tissue.

Ceramics are also widely used in dental applications and are currently been examined in bone tissue engineering. Two common ceramics used in dentistry and hip prosthesis include tricalcium phosphate (TCP) and hydroxyapatite (HA)[55,56]. Hydroxyapatite is a calcium-phospahate based ceramic and is the major component of the bone. The degradation of hydroxyapatite can be controlled by varying the chemical structure. In a recent study, hydrooxyapaptite-chitin material was implanted into the intramusculature of a rat model [52]. These materials were found to be non-cytoxic and degraded in vivo. Further, freeze-dried HA-chitin were used as scaffolds [52] and MSCs from NZW rabbits were induced into osteoblasts in vitro using dexamethasone. One of

the disadvantages of the calcium based ceramics is the low mechanical strength of these porous materials. In another study heat treated porcine tubular bone blocks were used for in vitro osteogenic differentiation of bone marrow stromal cells [53].

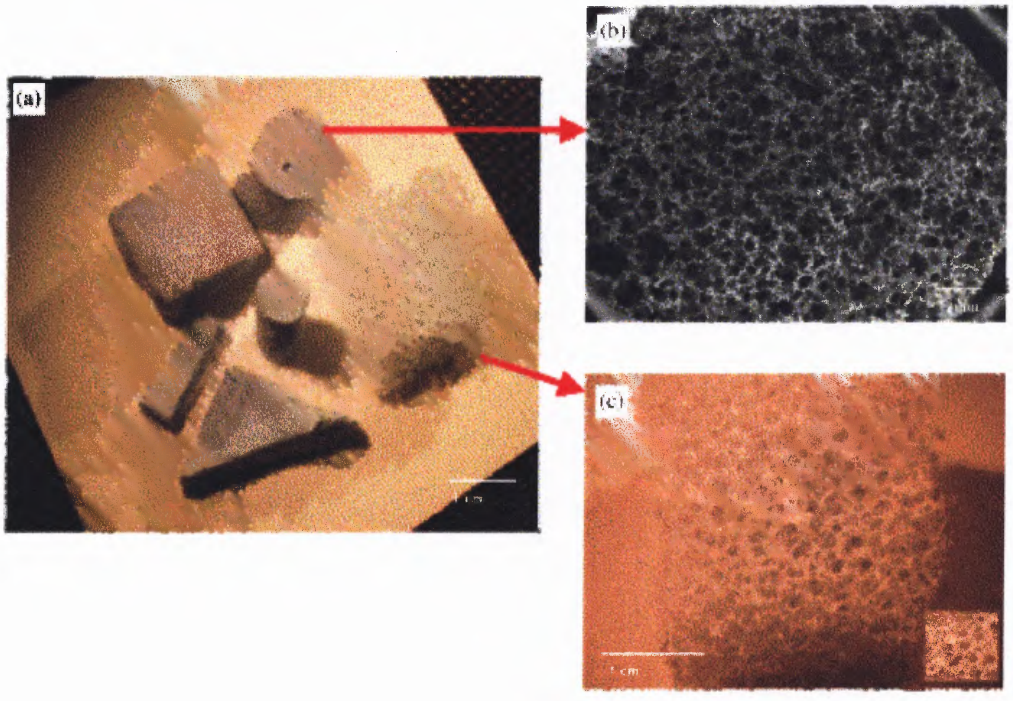


Figure 2.4 Optical microphotographs of (a) HA scaffolds of different shapes; (b) and (c) macroporous structures of HA scaffolds fabricated using combined gel-casting and polymer sponge methods[69].

Bioactive glass is another biodegradable material that has been recently studied in vitro [57] and in vivo [58]. The term bioactive refers to synthetic materials that form an interfacial bond with tissues upon implantation and enhance bone tissue formation as a result of surface modification when exposed to interstitial fluids. Studies indicate that bioactive ceramics can be used as a promising scaffold material for tissue engineered bone repair.

2.3 Polymers used in Study

In search for new biomaterials for specific applications scientists have developed some custom made biomaterials. Among the few are tyrosine derived polyarylates and polycarbonates, also referred to as pseudo polymers. The synthesis of pseudo polymers is based on the polymerization of tri-functional amino acids or dipeptides using the functional groups located on the amino acid side chains (62,63).The synthetic approach used for the preparation of tyrosine-derived polycarbonates and polyarylates give rise to polymer families allowing for systematic variations in pendent-chain and/or backbone composition (Figure 2.4 and 2.5). The alkyl esters of desaminotyrosyl-tyrosine-namely ethyl, butyl, hexyl, and octyl are used in the synthesis of the polycarbonates and polyarylates to efficiently vary the pendent chain. In addition the tyrosine derived polyarylates offer the ability of backbone variations by selecting diacids with varying numbers of methylene groups. Structural modifications of the pendent chain and the backbone provide a convenient way to modify the polymers and create polymer families whose members exhibit systematic and gradual changes in the thermal behavior, mechanical properties, degradation, hydrophobocity / hydrophilicity, and cell-polymer interaction [65].

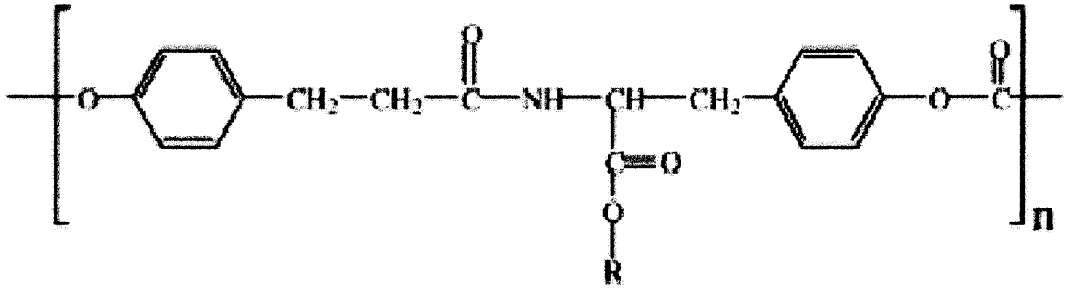


Figure 2.5 Chemical Structure of Tyrosine Derived Polycarbonate[64].

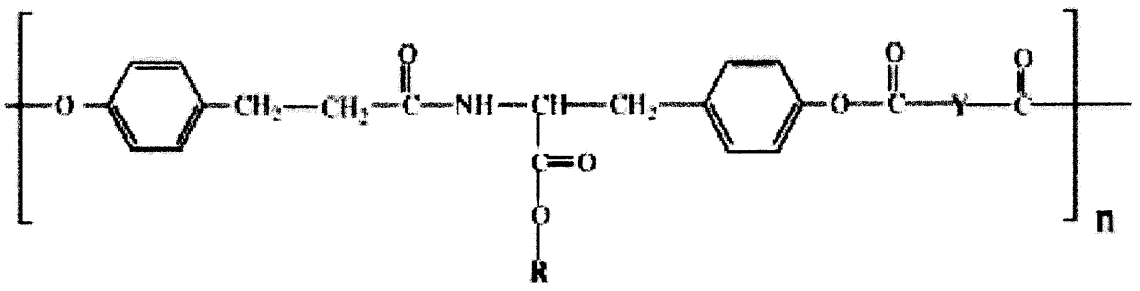


Figure 2.6 Chemical Structure of Tyrosine Derived Polyarylates [64].

These pseudo poly (amino acids) are different from most conventional poly (amino acid) in that they are soluble in organic solvents and are completely amorphous. While conventional poly(amino acids) are highly ordered, the disruption of the regular peptide backbone by non amide linkages reduces the inter- and intra-molecular chain forces, imparting solubility and amorphous character to pseudo-poly(amino acids). For tissue engineering applications to produce open polymer scaffolds of polyarylates and polycarbonates, a successful solvent casting/ salt leaching techniques have been evaluated [66].

Tyrosine derived polycarbonates are a group of homologous carbonate-amide copolymers differing only in the length of their respective alkyl ester pendent chain. They are characterized by their relatively high strength (50-70 MPa) and stiffness (1-2 GPa). The strongest and the stiffest polymer is poly (DTE carbonate). Polymer properties such as glass transition temperature, surface properties, and mechanical properties, can be easily controlled by varying the length of the alkyl ester pendent chain (Table 2.1). The physicomechanical properties and potential applications of tyrosine derived polycarbonates were studied by Ertel and Kohn [67]. Briefly, the polycarbonates are amorphous polymers. Because of their high hydrophobicity, they do not swell in aqueous

media or during the degradation process. Glass transition temperature range from 52 to 93°C and decomposition temperature exceed 290°C providing a wide temperature window for thermal processing. Even in an unoriented stage (thin solvent cast or compression molded films), they maintain their high mechanical strength and stiffness [67].

Table 2.2 Properties of Tyrosine-Derived Polycarbonates[67].

Polycarbonate derived from	**Glass Transition Temp (°C)**	**Contact Angle (deg)**	**Decomposition Temperature (°C)**
DTE	81	73	290
DTB	66	77	290
DTH	58	86	320
DTO	53	90	300

The family of polyarylates, however exhibit a range of physical properties, since the chemical structure of tyrosine-derived polyarylates can be modified both in pendent chain and polymer backbone. The combinatorial approach used in design of polyarylate library was described in detail by Brocchini et.al [68]. They are strictly A-B type copolymers in which the first monomer (A) contain a reactive group for attachment of a series of pendent chain, while the second monomer(B) allows for the systematic variations in the polymer backbone structure. Figure 2.6 and 2.7 describe the correlation between the polymer structure and selected polymer properties in a family of polyarylates [65].

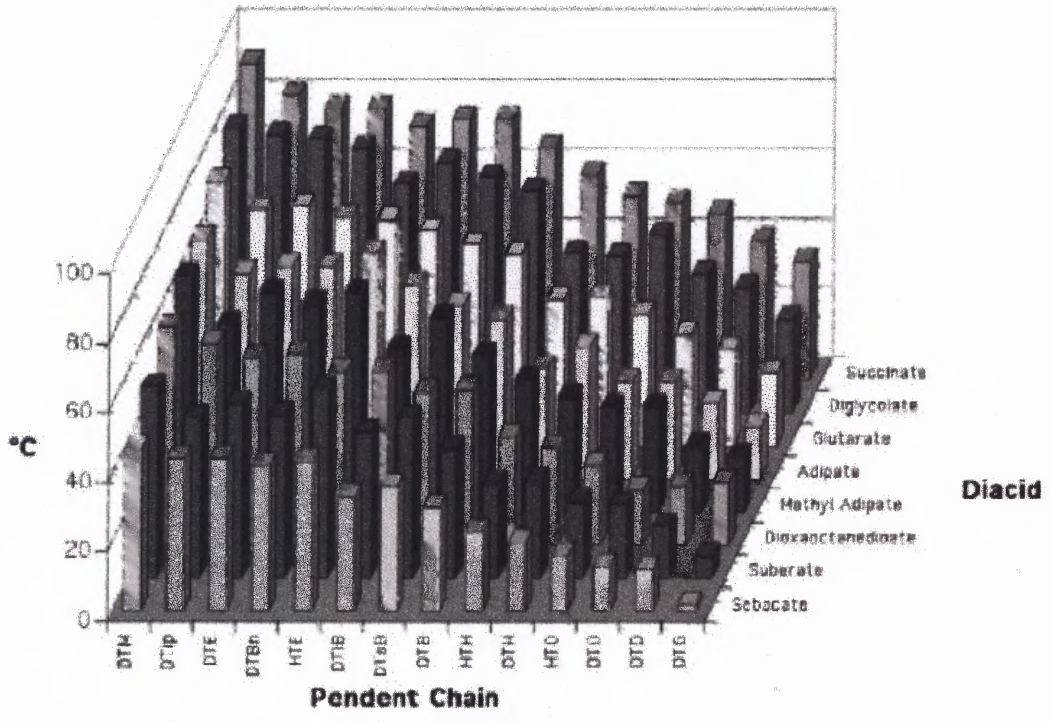


Figure 2.7 Pattern of Glass Transition Temperatures in the Family of Polyarylates [71].

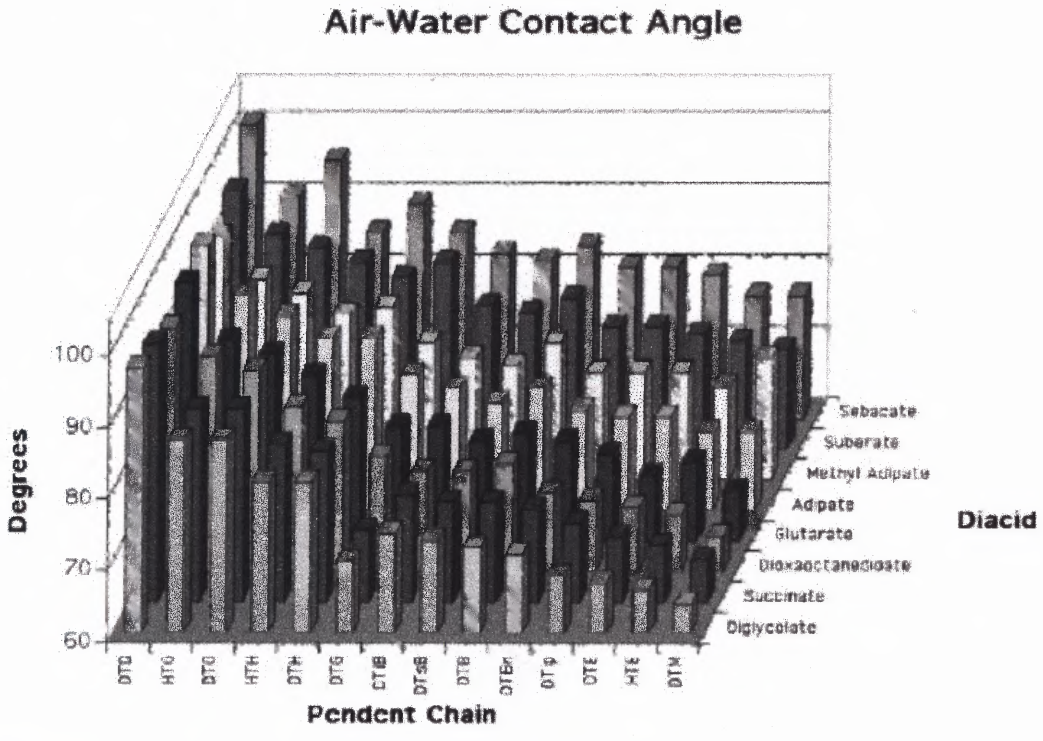


Figure 2.8 Pattern of Air-Water Contact Angle in the Family of Polyarylates[71].

The term "glass transition" temperature (Tg) is the temperature below which molecules have very little mobility. On a larger scale, polymers are rigid and brittle below their glass transition temperature and elastic above it. Tg is usually applicable to amorphous phases and is commonly applicable to glasses and plastics.

Air-water contact angle is a measure of hydrophobicty / hydrophilicity. The figure 2.7 indicates that as the pendent chains are increased from ethyl to octyl, the hydrophobicity increases.

The polymers used in the study were from the groups of polyarylates and polycarbonate. They were chosen because of their varying surface properties. The table 2.2 provides the list of polymers chosen in the study with their surface properties and glss transition temperatures.

Table 2.3 Properties of Polymers used in the Study.

Polymer	**Water Contact Angle (deg)**	**Glass Transition Temperature (°C)**
P(DTE) succinate	68	75
P(DTE) carbonate	73	81
P(DTE) sebacate	78	38
P(DTO) sebacate	95	10

2.3 Statement of Objective

Most of the biomaterials currently investigated as scaffolds for MSCs have been largely by trial and error testing using in vitro culture and/or animal models. Among all the polymers PLA-PGA are the widely studied and used biodegradable polymers used in the field of tissue engineering. However these materials have shown to elicit some foreign body response in many patients. [50, 51]. This limited supply of raw materials presses a need for more biomaterials. In an attempt to increase the library of biomaterials, a group at Rutgers headed by Joachim Kohn have created a combinatorial library of tyrosine derived polyarylates and polycarbonate [64]. They have also studied the ability of fibroblast and dorsal root ganglion cells to attach and grow on polycarbonate surfaces as a function of the surface hydrophobicity [64]. Their results indicate that rat lung fibroblast prefer relatively hydrophilic surfaces, and their ability to attach and grow on tyrosine derived polycarbonates can be controlled by the length of the pendent chain of the polymers. However the attachment of chick embryo dorsal root ganglion cells indicates a preference for the more hydrophobic surfaces. Previous studies also confirm that the cellular response to a polymeric scaffold can be influenced by slight variations in the material substrate (59) It was noted that the rate of fibro vascular tissue infiltration increased as the crystallinity of the PLA scaffold decreased (60). Such material-dependent results are also supported by Park and Cima (61) who reported that slight changes in PLA crystallinity across a 13-37% range influenced the behavior of hepatocytes and fibroblasts in vitro. Such interesting materials dependent phenomena need to be better understood since tissue engineering relies on the precise manipulation of associated cellular response.

As a first step towards developing substrates for stem cell therapies, this study investigates the osteogenic differentiating capability of human mesenchymal stem cells on the tyrosine derived polyarylates and polycarbonate. By modifying the pendent chain and backbone in these polymers, they exhibit systematic and gradual changes in thermal behavior, mechanical properties, degradation, hydrophobicity / hydrophiilicity, and cell polymer interaction [65]. This study attempts to correlate the surface property of the polymers (hydrophobicity / hydrophilicity) to the osteogenic differentiating ability of the human MSCs. The polymers were processed using the solvent casting technique which has been described earlier [67]. The human MSCs were isolated from the bone marrows according to the technique described by Jaiwal et.al [68] and their differentiation into the bone cell phenotype was performed on the polymeric surfaces. The polymers used in the study were poly(DTE)sebacate, poly(DTO)sebacate, poly(DTE) succinate and poly(DTE) carbonate, which is currently being evaluated clinically for orthopedic applications. These polymers were compared with the most commonly used biopolymer poly (L lactic acid) and tissue culture polystyrene plate, which served as controls.

CHAPTER 3

EXPERIMENTAL PROCEDURES AND METHODS

3.1 Polymer Processing

The polymers obtained were in the amorphous form. The polymers were molded on the 96-cell culture polypropylene well plates. They were processed into thin films according to the technique described earlier [67]. Briefly, they were dissolved in methylene chloride to form a 1% solution of the polymers. They were then coated on the 96-well plate by pipetting out 300ul of the solution and allowing the solvent to air dry at room temperature for more than 48 hours. The polymers were characterized to verify the absence of methylene chloride and any structural changes due to processing by Thermogravimetric Analysis (TGA) and Differential Scanning Colorimetry (DSC) technique. The polymeric films samples for DSC and TGA were formed by using 1% polymer solution dissolved in methylene chloride and 1ml of the solution was pipetted out in polypropylene micro-centrifuge tubes. The samples were air dried at room temperature for more than 2 days. TGA and DSC were performed using TA instruments model Q100 and model Q50 respectively. The samples were tested using the heat-cold-heat method. TGA and DSC were performed on the polymers in molded form and the polymers in received state. The results were compared to observe any changes in the thermal properties of the polymers. The following section describes the principle of operation of the techniques used.

3.1.1. Thermogravimetric Analysis

TGA can be used for the determination of decomposition weight loss, combustion analysis, temperature stability, moisture content and reaction mechanism.

TGA measures weight changes in a material as a function of temperature (or time) under a controlled atmosphere. It can detect changes in weight of 1 μg. This is accomplished with an extremely sensitive balance hanging inside a furnace. A thermocouple mounted just a few millimeters from the sample pan ensures accurate temperature of the sample. The unit used in the research was from TA instruments, model Q50. 10mg samples of the polymers were prepared in a polypropylene micro centrifuge using the 1%solution for TGA and DSC. The results of the analysis are shown in the later chapter.

3.1.2 Differential Scanning Calorimetery

It is used for the determination of endothermic or exothermic reactions or phase changes of a sample. Several characteristics can be determined such as: melting point, curing temperature, reaction kinetics, freezing point, glass transition, physical transitions, heat capacity, reaction mechanism, kinetics, purity, product identification.

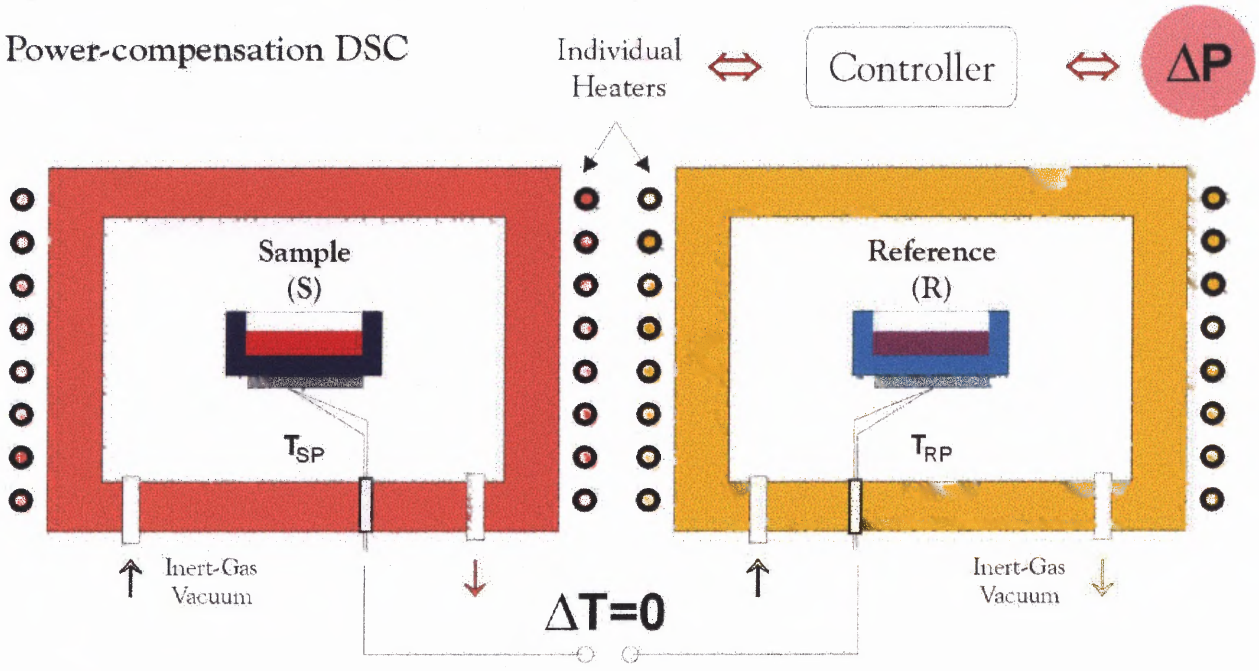


Figure 3.1 Schematic of Differential Scanning Colorimetry.

DSC measures temperatures and heat flows associated with thermal transitions in a material. It can detect changes in heat as low as 1 microwatt. This is accomplished with an extremely sensitive sensor array inside the heater cup. The heater cups have a weight less than 1 gm. This gives them a low thermal mass with extremely quick temperature response and extremely sensitive and accurate heat changes. The polymers were used using heat-cold-heat technique, in which the polymer is heated from 0 to 110 and then cooled to room temperature and then again heated to 110. The results are shown in the later chapter.

3.2 Cell Preparation

In this study the bone marrow from healthy human donors was purchased form Cambrex Inc. who refer to technique described in Neelam et.al. (41), in which fresh human bone marrow is obtained by routine iliac crest aspiration from normal human donors. The cells were then isolated according to previous protocols [41]. Briefly, 25ml marrow sample was washed with saline, followed by centrifugation over a density cushion. The interface layer was removed, washed and plated in tissue culture flasks in Dulbecco's Modifed Eagle Medium (DMEM)(Glaxo-BRC, Inc.) containing 10%fetal bovine serum(FBS) (Hyclone Inc.). Non-adherent cells were washed from the culture during biweekly feedings. Colony formation was monitored for a 14-17 day period. When the tissue culture flasks were near confluent, the MSCs were passaged. At the end of first passage, MSCs were enzymatically detached with 0.25% trypsin containing 1mM EDTA for 2-5min at 37°C, and subsequently replated for continued passaging. At the end of second passage, the MSCs were cryopreserved.

3.3 Media Preparation

The human MSCs isolated from the bone marrow were grown in the following medium.

Control Media (CM): The control media (CM) contains DMEM (low glucose), 10% fetal bovine serum and 1% antibiotic antimycotic. The media is filtered using the 0.45m cellulose acetate low protein binding membrane (Corning Incorporated)

Osteogenic Supplement (OS):OS media contains complete media with 1mM Dexamethasone (Dex), 1M β-Glycerophosphate (βGP) and 10mM ascorbic (AsAP) acid

3.4 Cell Culturing

The cryopreserved cells were thawed and seeded at a fixed density of $3x10^4$ cells/cm^{2i} in 96-well plates containing coated polymers and tissue culture plastic, which served as control.. The cells used were P1 (first passage) cells. The cells were grown in complete media in the presence or absence of OS for 14 days. The media were changed twice a week. Alkaline phosphatase activity, cell number by DNA quantification, and the amount of calcium in the extracellular matrix were measured at day 4, 8, 12 and 16. In addition, osteocalcin assay was performed at day 16. Cell were harvested from the 96-well plate using 0.1% triton X100 ( ) and Sigma cell lysates were then used for each assay as follows.

Basic principles of different assays used:

1. *Alkaline Phosphatase assay*: Alkaline Phosphatase (APase) is an enzyme that is present in osteoblasts, which plays an important in the role of mineralization. In this assay, the presence of AP is measured by the hydrolysis of p-nitrophenylphosphate (p-Npp) to p-nitrophenol (p-Np), which is read by an absorbance plate reader at 410 nm. Cell lysate in 0.1% Triton- X was used in the assay and the alkaline phosphates activity was measured using reagents from Sigma Kit (104) from Sigma Diagnostics and modified for 96-well plate absorbance spectrophotometer plate reader..
2. *DNA assay*: This assay is used to determines the DNA content in a sample. This DNA content is directly proportional to the cell number.In this study the Picogreen® dsDNA reagent (Molecular Probes- PicoGreen® dsDNA Quantitation Kit, Catalogue No: P-7589) was used to quantify the cell number.

PicoGreen® dsDNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double- stranded DNA (dsDNA) in solution. Detecting and quantitating small amounts of DNA is extremely important in a wide variety of biological applications. The concentration of dsDNA is determined by the absorbance of the sample using fluorometers or fluorescence microplate reader. In this study the fluorescence was measured using the Picoflour (Model 8000-003) fluorometer with blue light.

3. *Calcium Assay*: This assay is used to determine the calcium bone mineral content in the ECM. The samples were suspended in 0.5N HCl to dissolve the ECM and calcium content was detected using the Calcium Kit-587. If the assays were not performed the same day, the samples were stored at -20°C. In this study, the author has used the ThermoTrace: CALCIUM REAGENT, Cresolphthalein complexone(CPC) method of Moore and Briggs [70]. CPC reacts with calcium (Ca) and Mg magnesium in alkaline solution to form a deeply colored complex. 8-hydroxyquinoline is incorporated into the reagent to preferentially bind and revent interference from this cation. The intensity of the purple colored formed is proportional to the calcium concentration and is measured photometrically at 570nm using the Molecular devices Microplate Reader (Model- EMax)
4. *Osteocalcin assay*: Osteocalcin, the vitamin K-dependent protein of bone, is a specific product of the osteoblast. While osteocalcin is primarily deposited into the extracellular matrix of bone, a small amount can be detected in the blood. The assay measures only intact osteocalcin, which is synthesized de novo by the osteoblast, and it eliminates any potential confounding interference by circulating

fragments. Cell lysate suspended in 0.1% Triton-X was used and the assay was performed using the Intact Human Osteocalcin EIA Kit, Catalog no: BT-460 and the absorbance were measured at 450nm using the Molecular Devices Microplate Reader (Model- EMax).

3.5 Statistical Analysis

Statistical analysis was performed to evaluate the effect of different polymeric scaffolds on cell proliferation and differentiation. One-way analysis of variance (ANOVA) was performed between groups, for each culture media at each time point. Differences between groups were considered significant at a level of 0.05 *($p<0.05$).* Multiple comparisons between groups were performed by student t-test at each time point.

CHAPTER 4

RESULTS AND CONCLUSION

4.1 Results of TGA and DSC analysis

These techniques were employed to verify the absence of methylene chloride in the polymeric film. From the TGA results, it was observed that the weight loss in polymers as received and in the molded state is less than 1%, which suggests the presence of very little (less than 1%) methylene chloride. DSC graphs confirm the amorphous nature of the polymers since no crystallinity peaks are observed in the graphs. Moreover it can be observed that the there is no change or very little change in the glass transition (Tg) in polymers in the received state as compared to the polymers in the molded form. The figure 4.5 shows the result of the TGA run on poly (DTE) succinate.

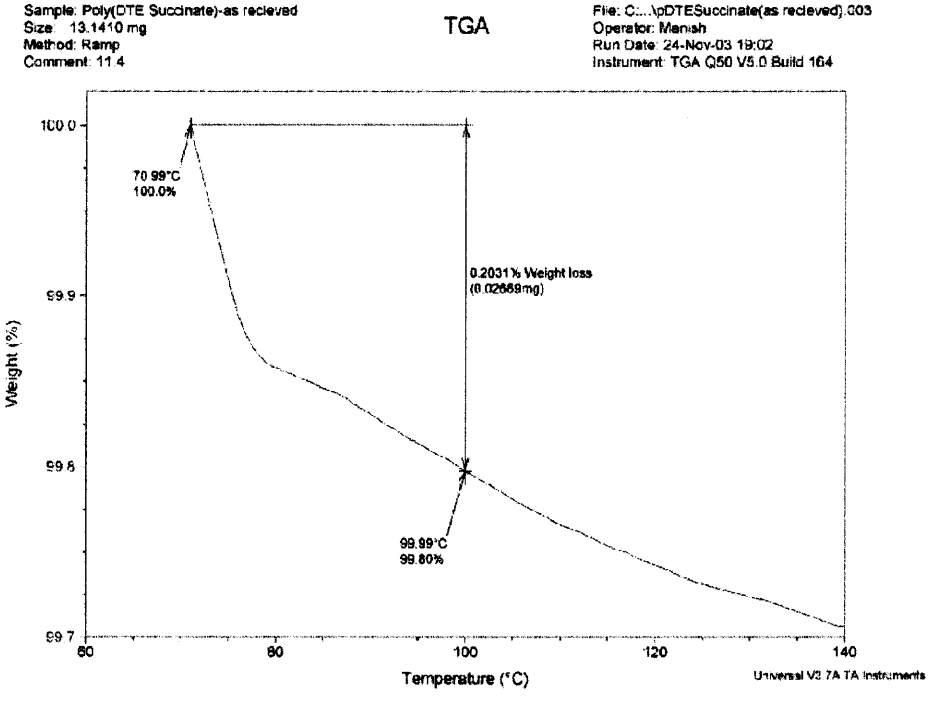


Figure 4.1 Results of the TGA run on poly(DTE) sebacate as received.

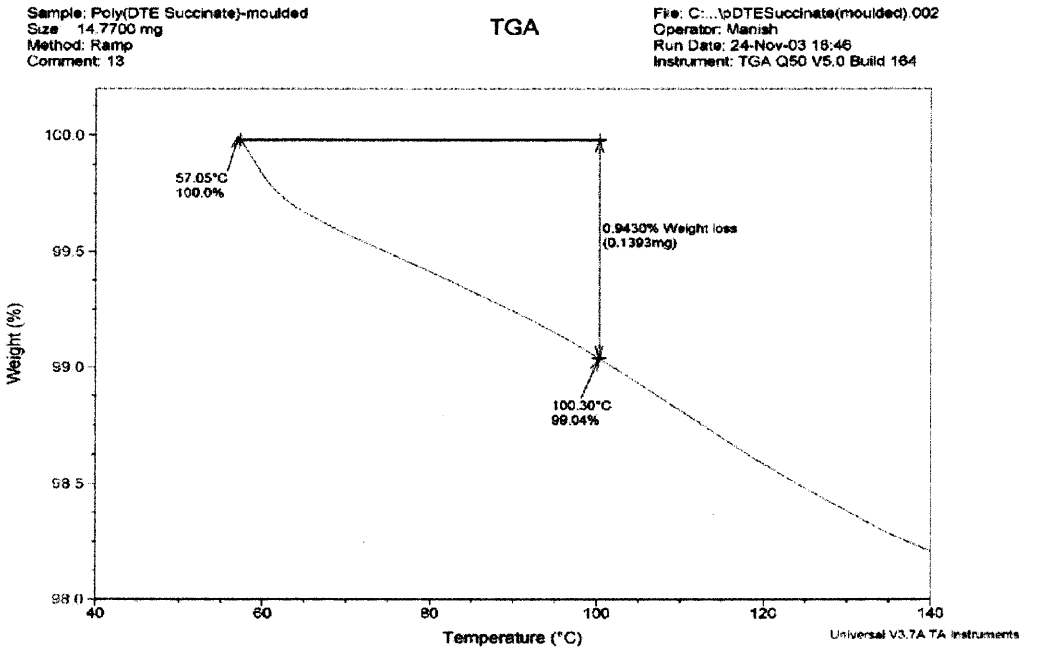


Figure 4.2 Results of the TGA run on poly(DTE) sebacate in the molded form.

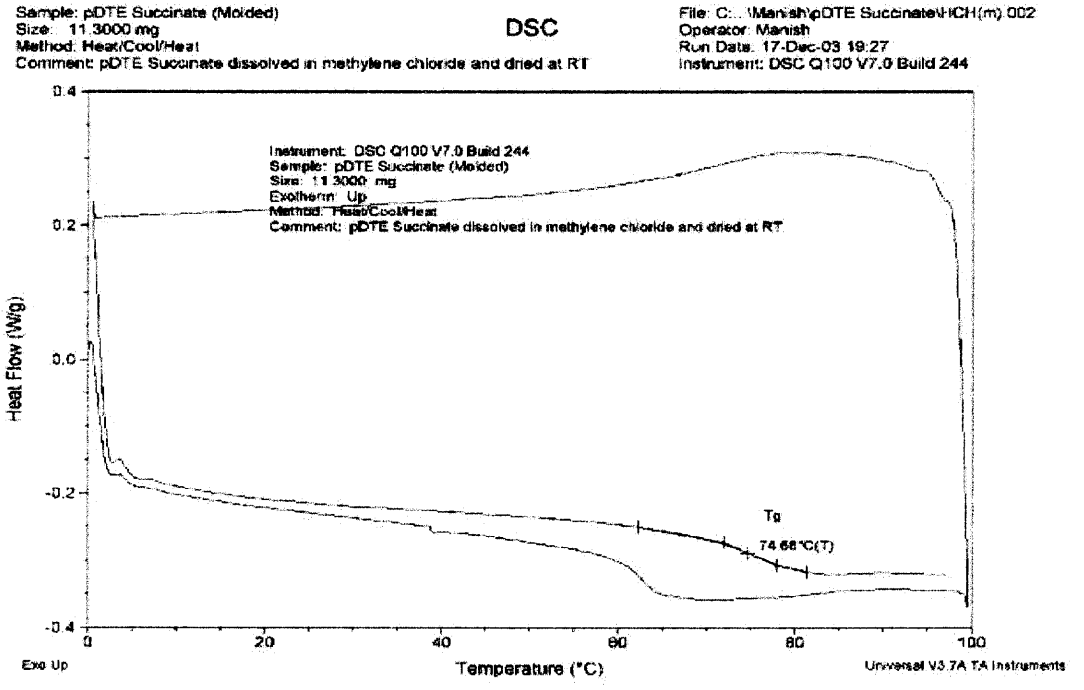


Figure 4.3 Results of the DSC run on poly (DTE) Succinate in the molded form.

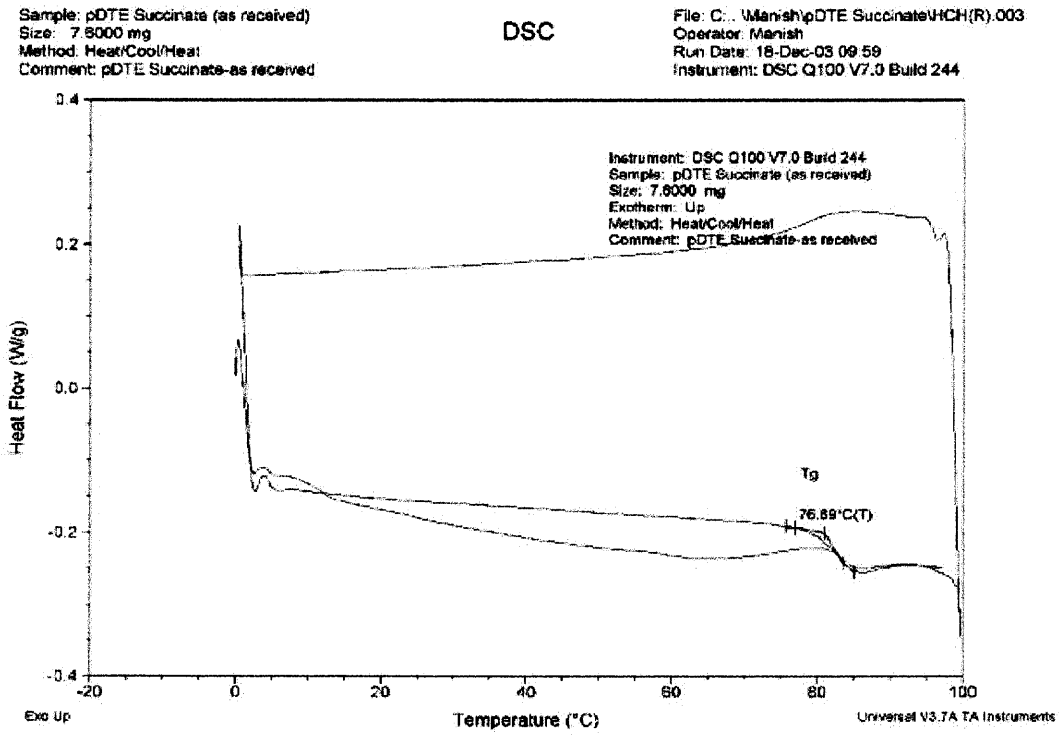


Figure 4.4 Results of the DSC run on poly (DTE) Succinate as received.

4.2 Result of DNA Assay

The polymers were arranged in increasing order of their hydrophobic property on the 96 culture well plate. The seeded cells were then washed with DI water to completely remove the media and suspended in 60µl of 0.1% Triton-X solution for 30 minutes to lyse. 25µl of the lysate was used for the assay and the concentration of cells was determined by the measuring the absorbance of the sample using fluorometer Picoflour (Model 8000-003).The results of the DNA assay on day16 are shown in figure 4.1 and is also printed in appendix A.. The results of day 4,8 and 12 are also present in appendix A

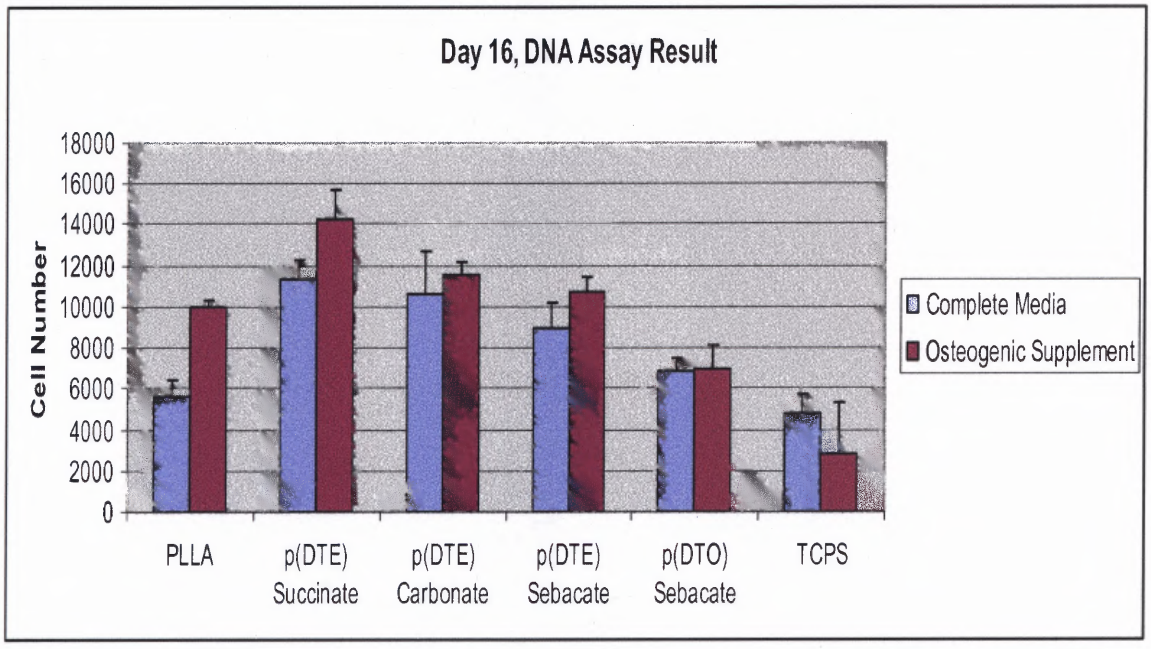


Figure 4.5 Graph showing cell proliferation on different polymeric scaffold. Values are Mean± SD, n=4 per time point. *($p<0.05$).*

From the graphs, it was observed that the cells grown on pDTE succinate in OS proliferated significantly higher than all other polymers in the group($p<0.05$). It was also observed that the proliferation decreased from the most hydrophilic polymer, pDTE succinate, to the most hydrophobic polymer pDTO sebacate.

4.3 Result of Alkaline Phosphatase Assay

Cell lysate in 0.1% Triton- X was used in the assay and the alkaline phosphates activity was measured using reagents from Sigma Kit (104) from Sigma Diagnostics and the McKay Laboratory Alkaline Phosphatase Assay for 96-well plate absorbance spectrophotometer plate reader.(Micro Devices Microplate Reader, Model –Emax.

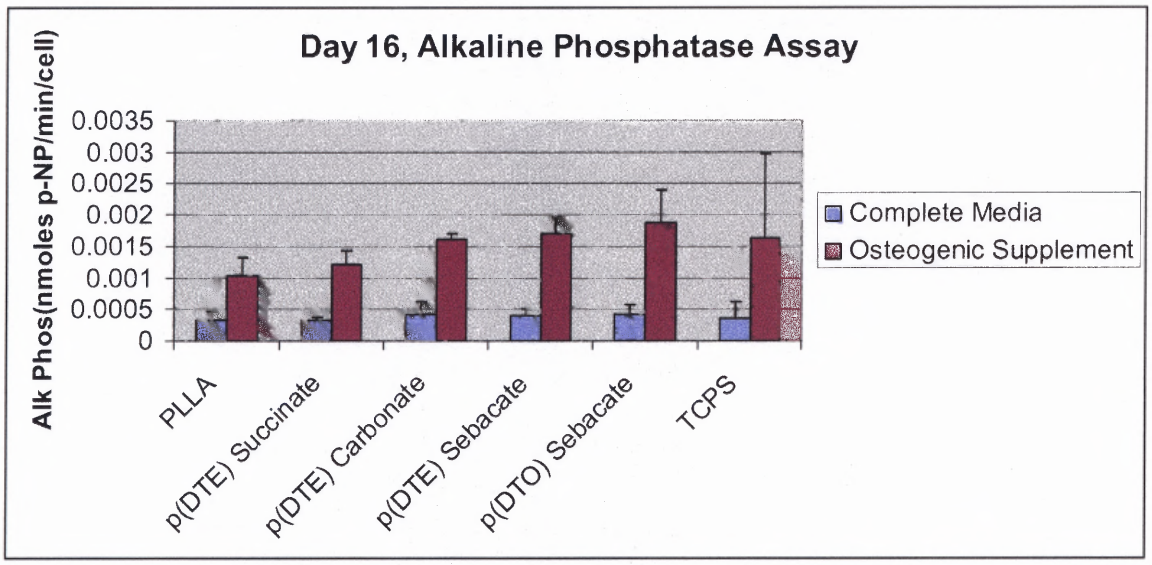


Figure 4.6 Results of the Alkaline Phosphatase Assay. Values represent Mean SD, n=4 per time point. *($p<0.05$).*

From the figure 4.2, it can be observed that, for MSCs grown in the presence of OS, the alkaline phosphatase (APase) activity is significantly higher than in complete media.. Also the figure suggests that the APase activity is the highest in case of poly(DTO) sebacate ($p<0.05$). The results of the APase assays performed at day 4, 8 and 12 are shown in the appendix B.

4.4 Result of Calcium Assay

The frozen samples were thawed at room temperature and kept on the shaker for 24 hours. The samples were centrifuged for 2 minutes and 20 of the samples were collected from the supernatant. The assays were then performed using the regeants in the Calcium kit. The results of the assay performed at day 16 is shown in the figure 4.3. The results of the assays done on day 4,8 and 12 are present in the appendix C.

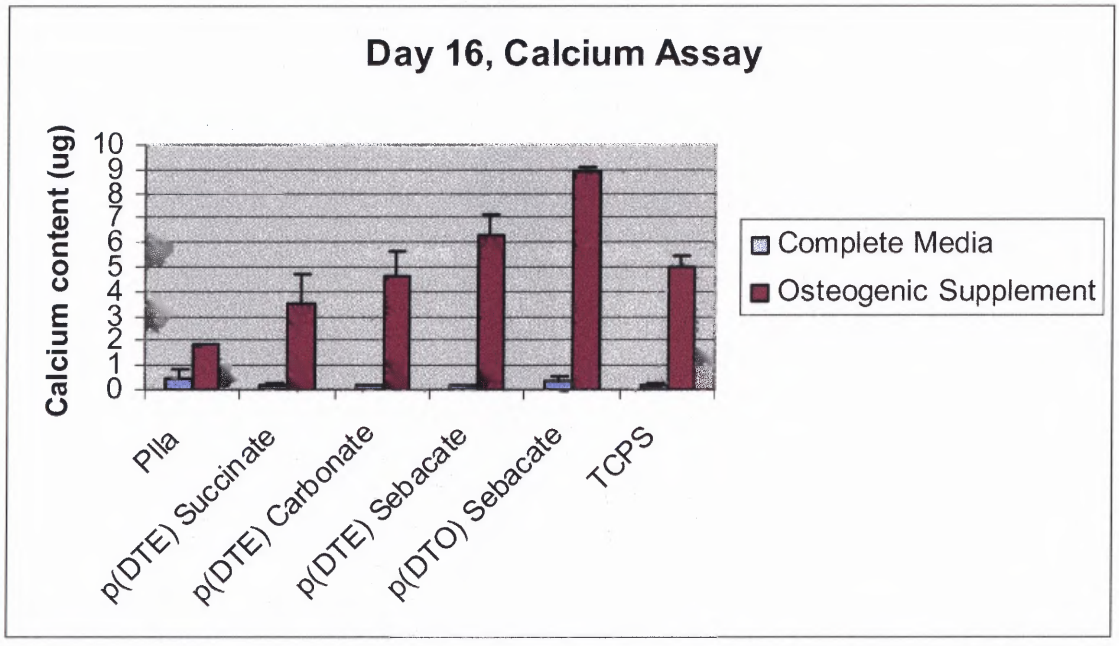


Figure 4.7 Results of Calcium Assay .Values are Mean ± SD, n=4. *($p<0.05$).*

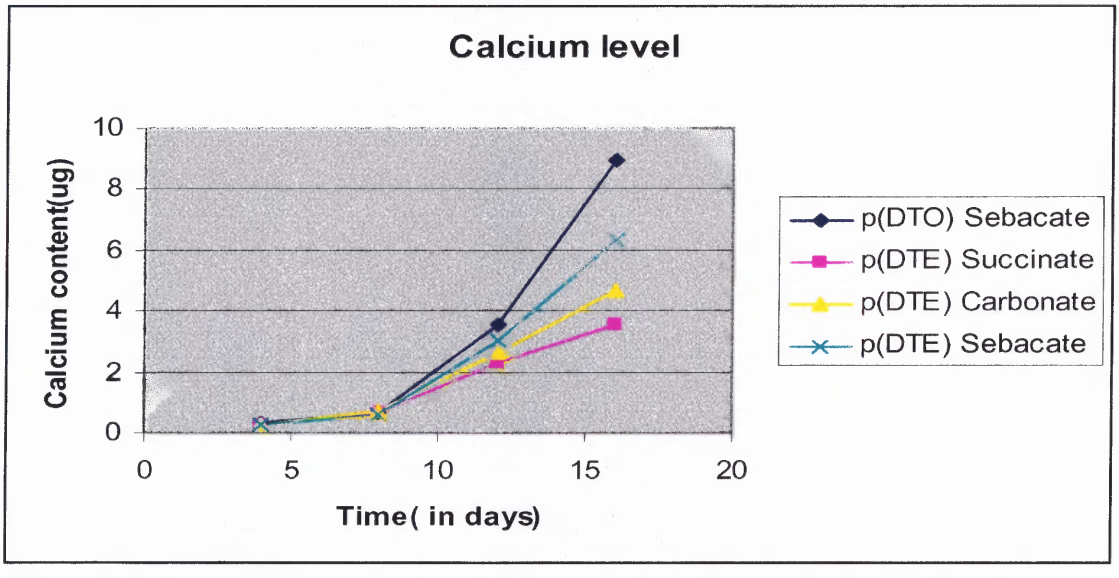


Figure 4.8 Calcium content on Day 4,8,12 and 16 on different polymeric scaffolds.

From the results it can be observed that the level of calcium is much higher in the samples grown in OS media than CM. Also it is evident that the level of calcium is highest in case of the most hydrophobic surface pDTO sebacate ($p<0.05$), confirming

that the MSCs differentiation towards the osteogenic lineage is enhanced by more hydrophobic polymers.

Also it was observed that the calcium content increased with time. Figure 4.4 shows the results of the time analysis of the cells grown in OS media on p(DTO) sebacate (most hydrophobic) and p(DTE) succinate (most hydrophilic).

4.5 Result of Osteocalcin Assay

The production of osteocalcin, a bone specific protein produced by late stage osteoblasts was determined by an enzyme immunoassay kit (Biomedical Technologies Inc.) at day 16 only. The results of the assay are shown in figure 4.4. Results indicate that the higher concentration of osteocalcin is found in more hydrophobic polymers, confirming that the human MSC differentiation along the osteogenic lineage is enhanced by more hydrophobic polymers

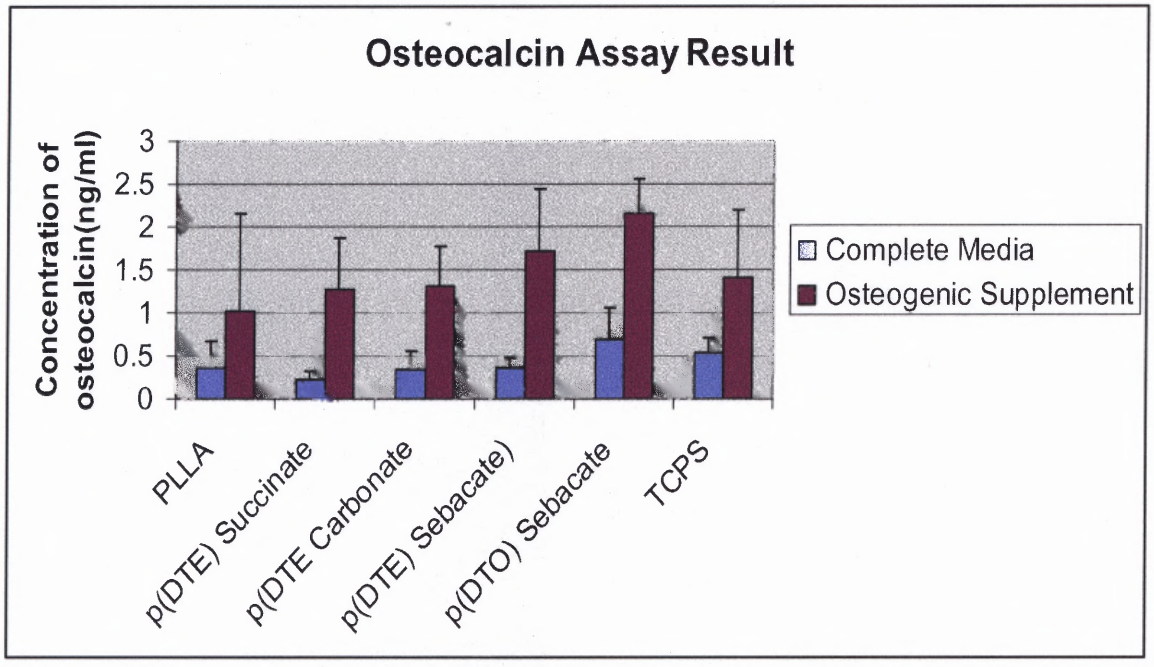


Figure 4.9 Results of Osteocalcin Assay. Values are Mean ±SD. *($p<0.05$).*

CHAPTER 5

CONCLUSION AND SUGGESTION FOR FUTURE RESEARCH

A summary of results of this research and recommendations for future work will be the focus of this chapter.

5.1 Summary of Results

From the TGA results, it can be confirmed that processing the polymers using the solvent casting technique does not alter the properties of the polymers. This work substantiates several important conclusions regarding the ability of the human MSCs. A major observation is that the human MSCs proliferate at a higher rate on hydrophilic surface. During the 16 day assay period, MSCs cultured in OS underwent a significant increase in the Apase activity, which can be interpreted to reflect the degree of progression into the osteoblasts. The subsequent decrease in the Apasa acivity per cell beyond day 12 correlates with the advanced matrix mineralisation, modulation of osteocalcin expression, and terminal osteogenic cell differentiation as MSCs become osteocytes. This study also confirms the ability of human MSCs to form osteoblasts in vitro.

Another observation is that alkaline phosphatase activity of the human MSCs is higher on hydrophobic polymers, suggesting that the more hydrophobic polymers enhance the osteogenic differentiation. This is a very contrasting observation as cells are believed to prefer hydrophilic surfaces. However, studies have shown that different cells behave differently on the same polymeric surfaces[65].

Previous studies have indicated that surface properties can influence the growth of cells[61]. Studies also indicate that small changes in polymer chemistry in tyrosine derived polycarbonates have a large effect on the bone-implant[59]. In this study, tyrosine derived polyarylates and polycarbonates are first time been developed as tissue engineering scaffolds for stem cell bone tissue engineering. This study demonstrates that the differentiation of the human MSCs towards the osteogenic lianeage can be influenced by the surface properties of the scaffolds. The results show that more hrdrophillic substratum stimulates the cell growth, however the most hydrophobic polymeric scaffold influenced the osteogenic differentiation of the human MSCs. Thus this study, for the first time demonstrated that human MSC differentiation along the osteogenc lineage could be influenced not only by hormones and growth factors present in the medium but also by the chemical structure of the underlying substratum.

5.2 Suggestions for Future Research

This research provides a strong basis for future research using human MSCs to treat bone disorders. In this thesis the author has touched upon the key role of the substratum in bone tissue engineering. Data collected from this research has shown, that using polymeric scaffolds with varying hydrophobicities can alter the response of stem cells. Preliminary testing has been helpful, however the handling technique needs to be refined. It is evident from the results that the cells scraped from the polymer during assay procedure cannot guarantee that 100% cells have been scraped out from the surface during sample collection. Moreover, while scrapping the cells from the surface, the

polymers tear and some cells may be lost during the sample collection. A better way to ensure sample collection would be to use a microscope to observe the surface while scrapping.

It would be of great interest to investigate the effect of varying cell density on the polymeric surface. This could be achieved by seeding cells at densities of 10000, 20000, 50000 cells/cm2 on to the polymeric surface.

It can of interest to observe the cell behavior to surface texture. Studies have shown that different cells types behave differently on rough and smooth surfaces. Rough surfaces increases hydrophobidity. Thus it can be hypothesized that surface roughness will further enhance the growth of osteoblasts.

Another area of research would be to test the ability of the human MSCs differentiation capability towards the chondrogenic lineage on these polymers. Since these polymers offer a gradual change in surface chemistry, it would of interest to verify whether the chondrocyte growth and differentiation can also be influenced by surface hydrophobicity.

Since these polymers are biodegradable and this thesis suggests that the MSCs could be differentiated into bone cells on these polymeric scaffolds, a major step in the future would be to test them *in vivo*.

APPENDIX A

DNA ASSAY TEST RESULTS

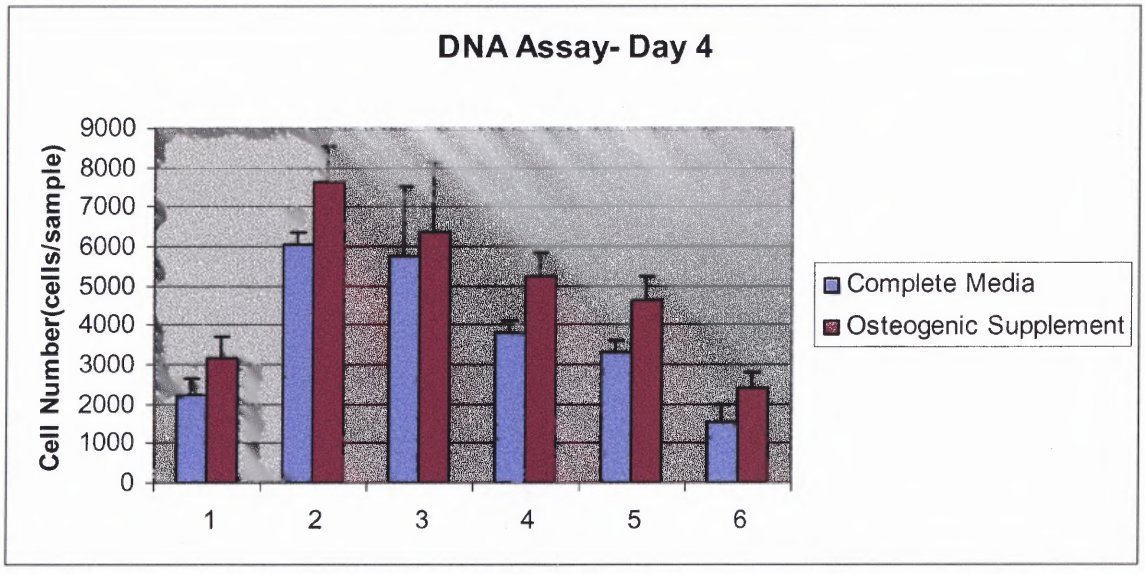


Figure A.1 Day 4 DNA Test results. Graph of cell number versus polymers.Values are Mean ± SD.*(p<0.05).*

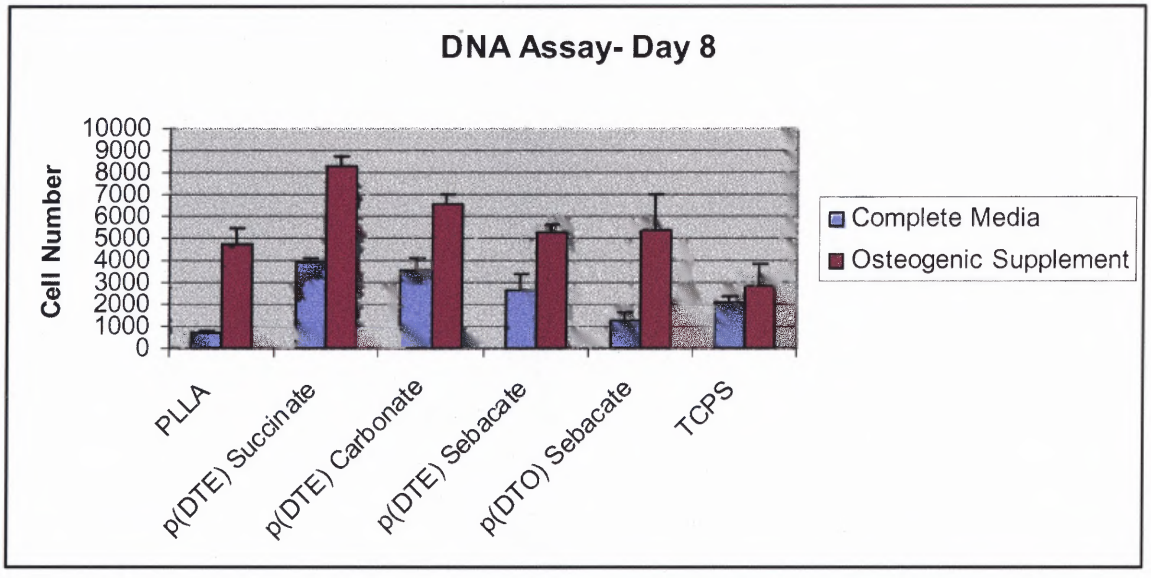


Figure A.2 Day 8 DNA Test Result. Values are Mean ± SD. *(p<0.05).*

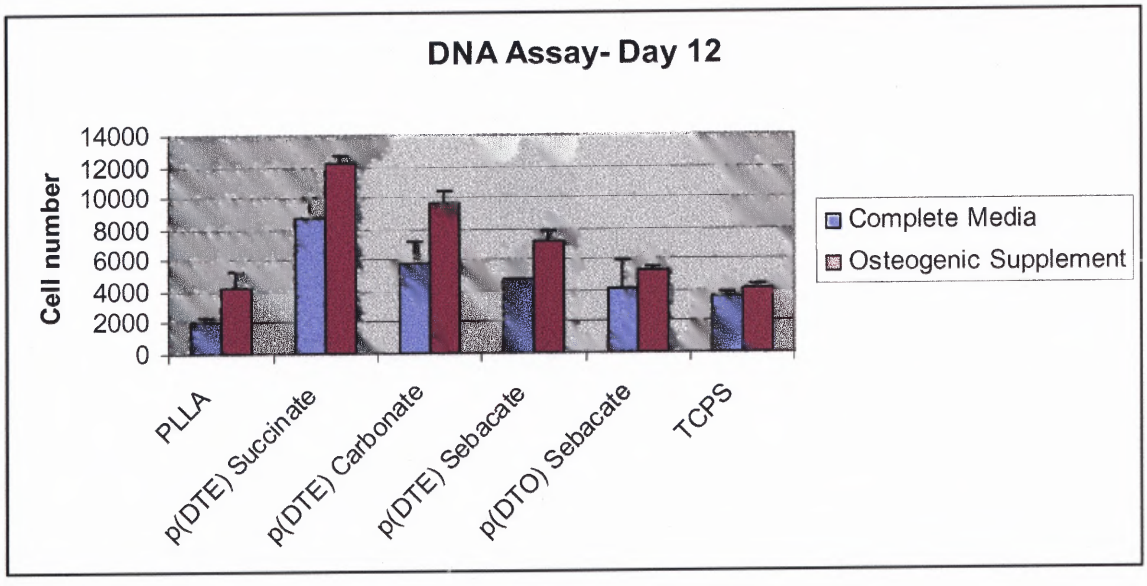


Figure A.3 Day 12 DNA Test Results. Values are Mean ± SD. *($p<0.05$).*

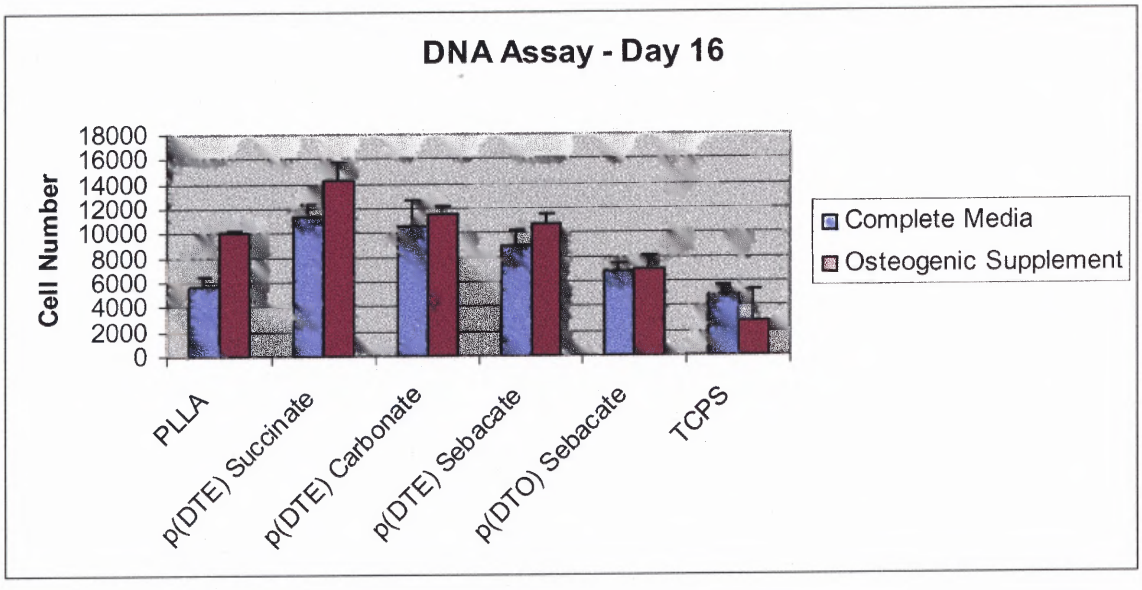


Figure A.4 Day 16 DNA Test Results. Values are Mean ± SD. *($p<0.05$).*

APPENDIX B

ALKALINE PHOSPHATASE ASSAY TEST RESULTS

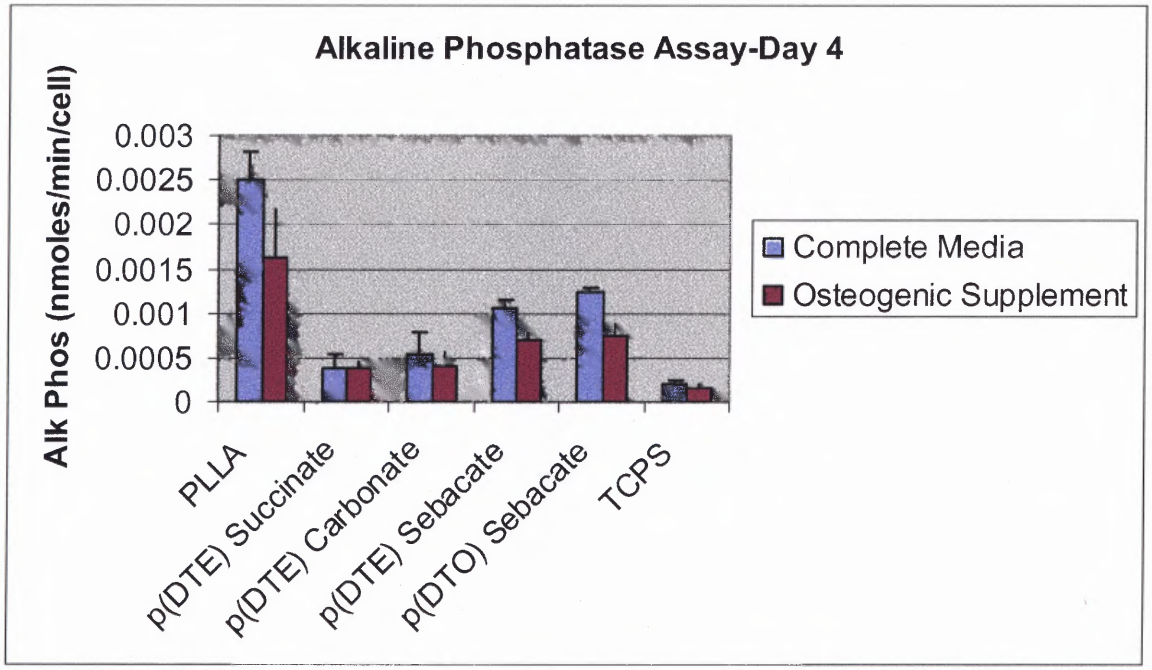


Figure B.1 Day 4 Alkaline Phosphatse Assay Test Results. Values are Mean ± SD.*(p<0.05).*

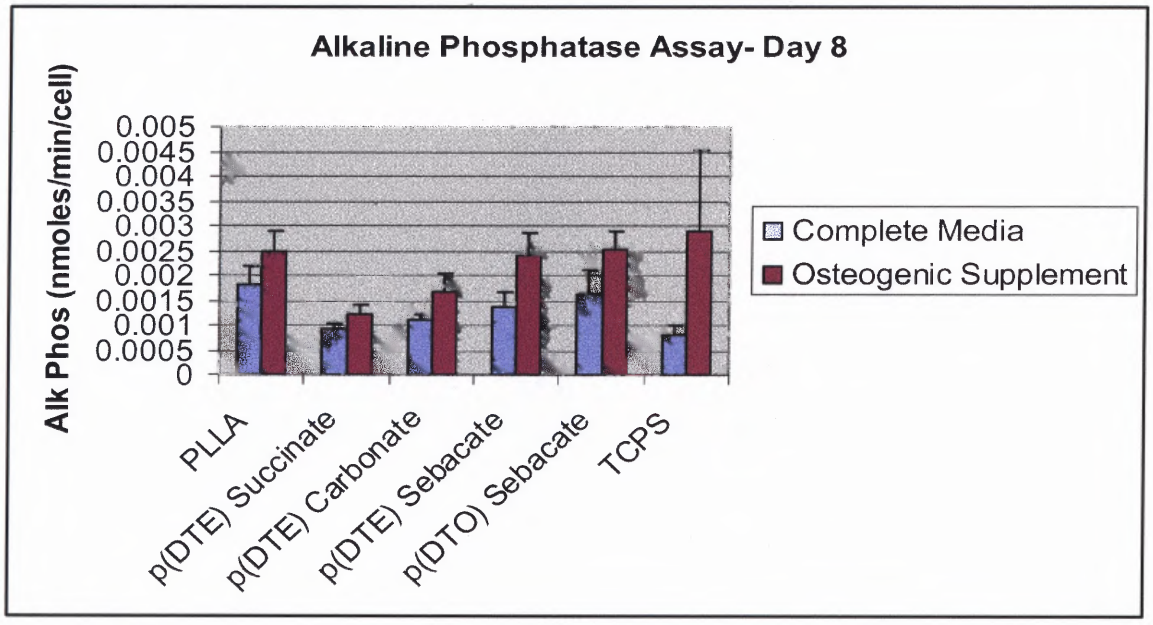


Figure B.2 Day 8 Alkaline Phosphatase Assay Test Results. Values are Mean ± SD. *(p<0.05).*

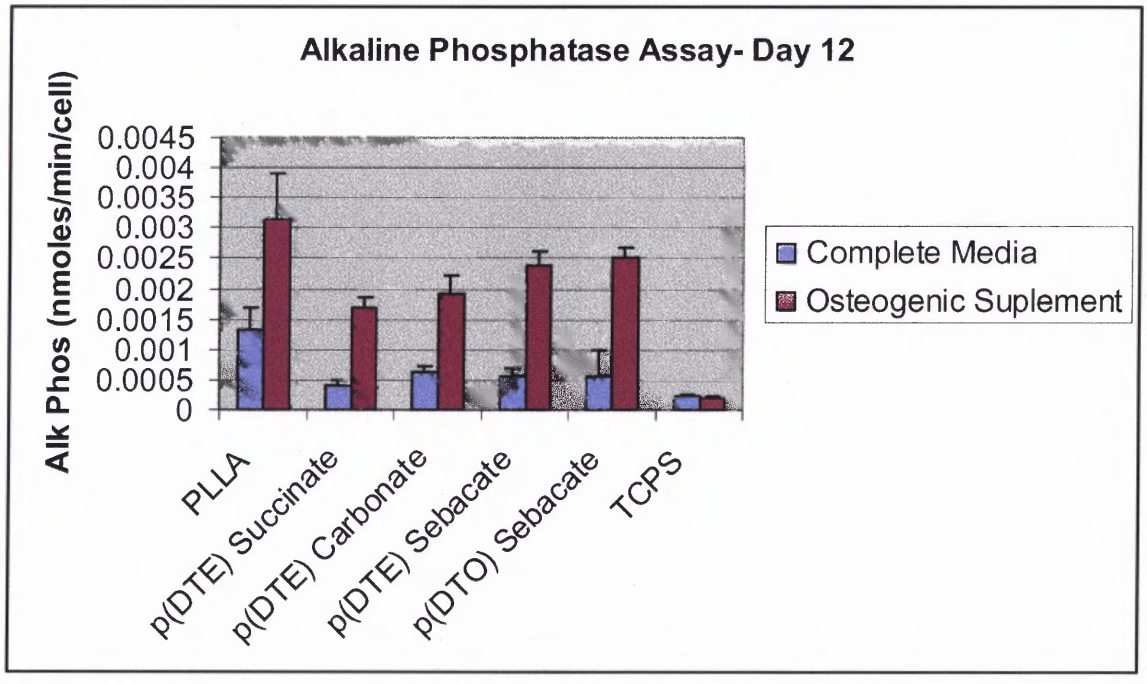


Figure B.3 Day12 Alkaline Phosphatase Assay Test Results. Values are Mean ± SD. *($p<0.05$)*

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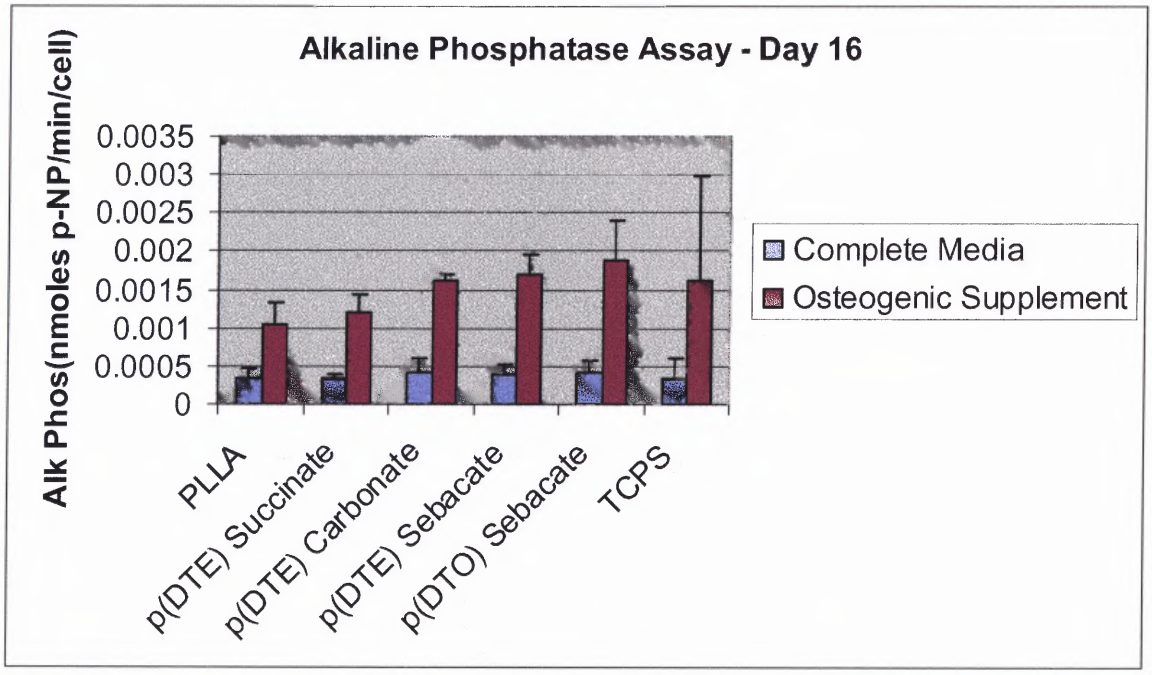

Figure B.4 Day 16 Alkaline Phosphatase Assay Test Results. Values are Mean ± SD. *($p<0.05$).*

APPENDIX C

CALCIUM ASSAYS RESULTS

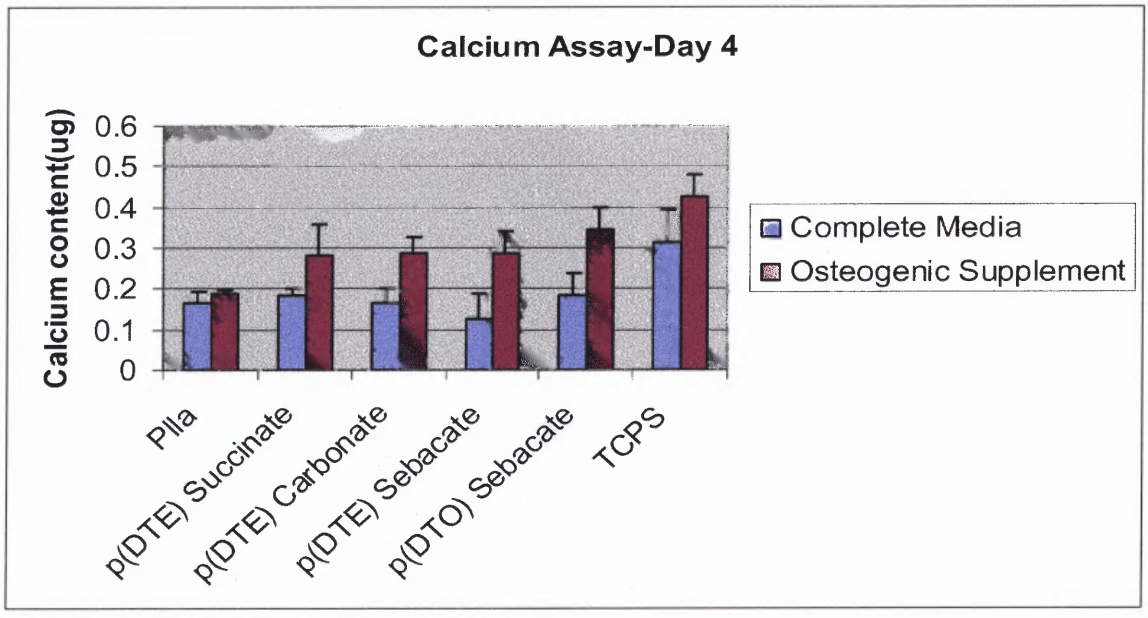

Figure C.1 Day 4 - Calcium assay result. Values are Mean ± SD. *($p<0.05$).*

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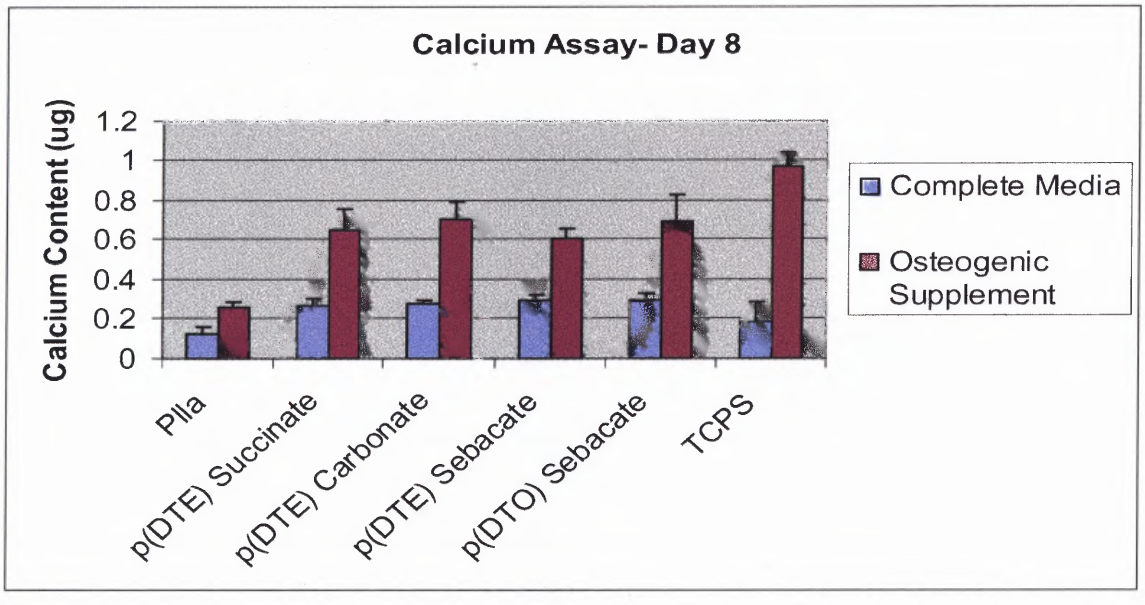

Figure C.1 Day 8- Calcium assay result. Values are Mean ± SD. *($p<0.05$).*

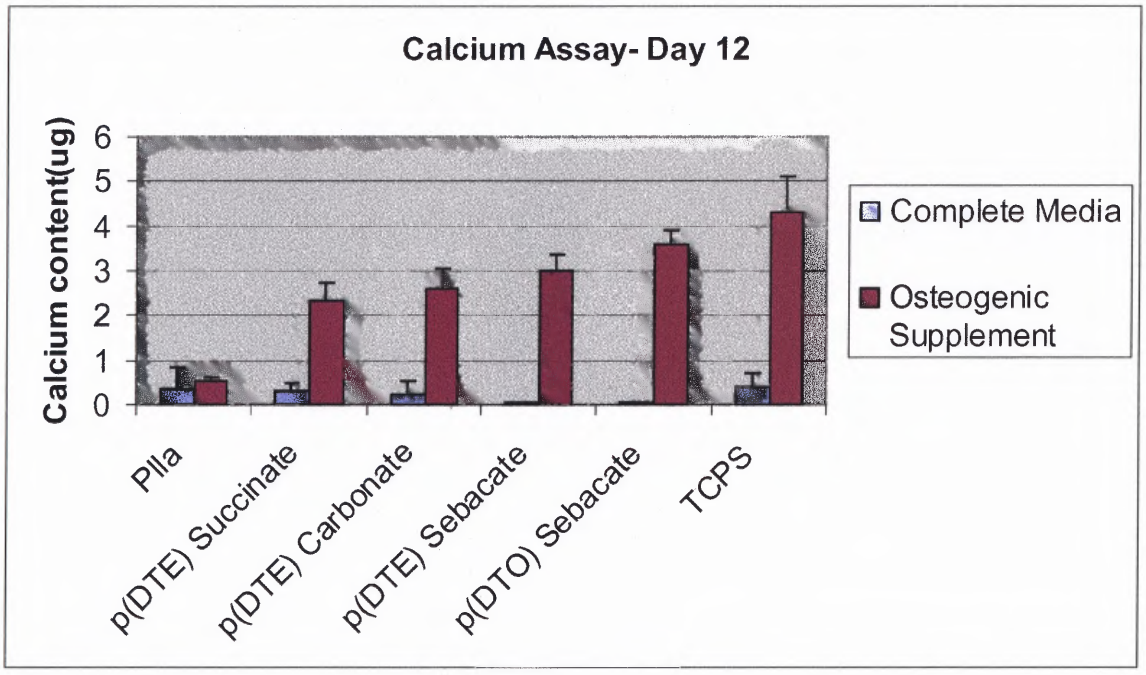

Figure C.3 Day 12- Calcium assay result. Values are Mean ± SD. *($p<0.05$).*

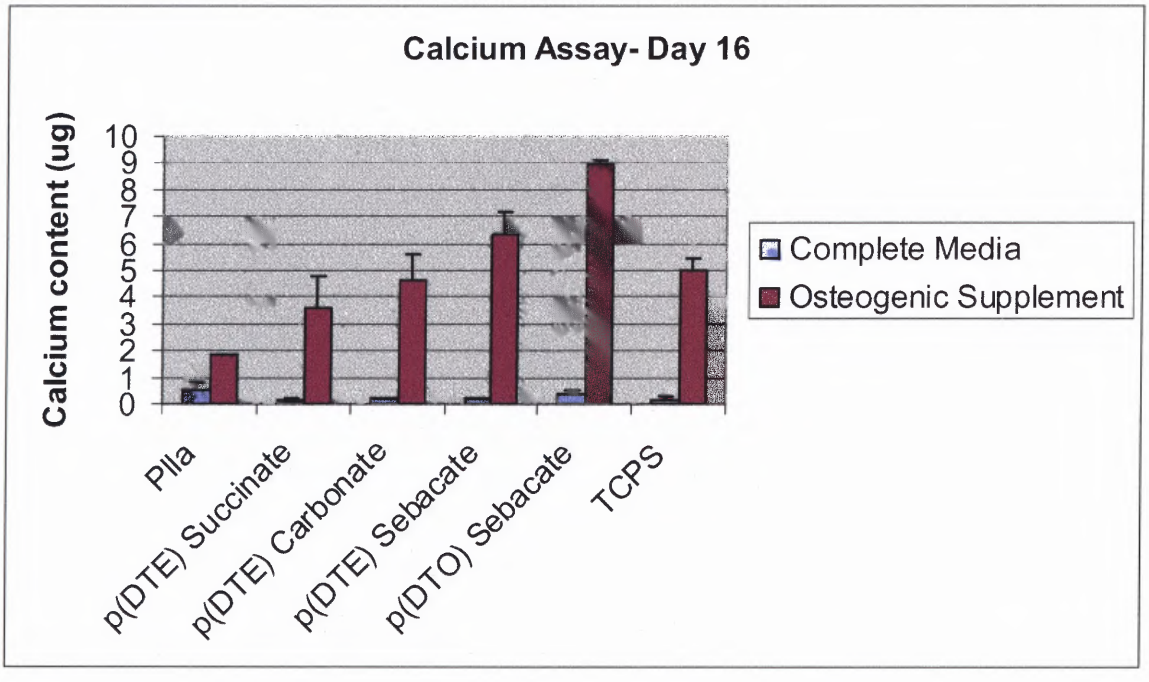

Figure C.4 Day 16- Calcium assay result. Values are Mean ± SD. *($p<0.05$).*

APPENDIX D

TGA TEST RESULTS

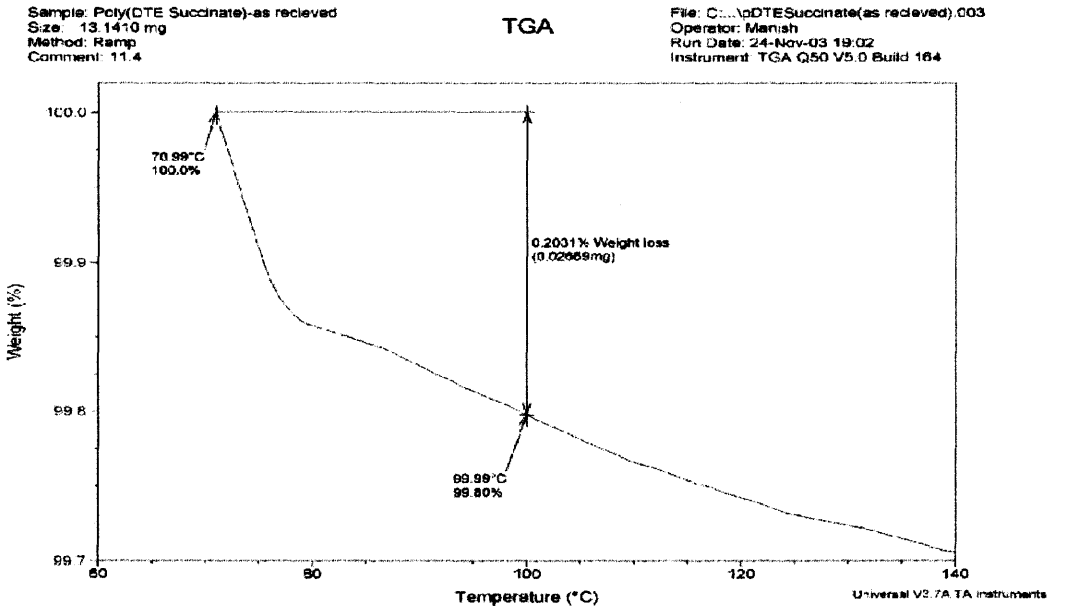

Figure C.1 TGA results of poly(DTE) Succinate in received form.

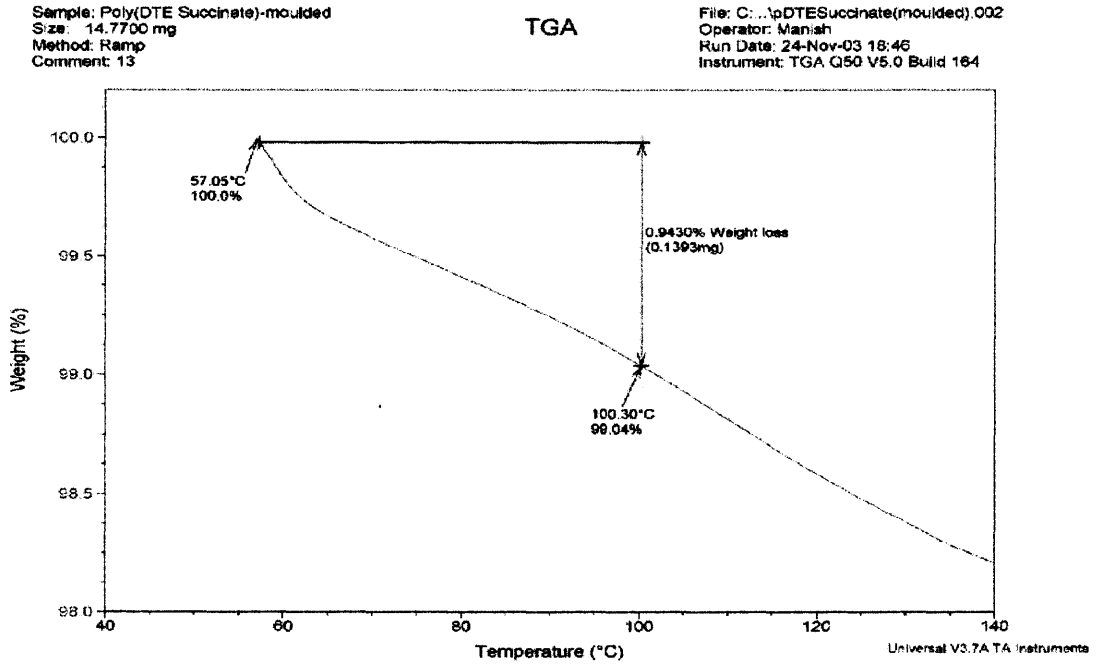

Figure C.2 TGA results of poly(DTE) Succinate in molded form.

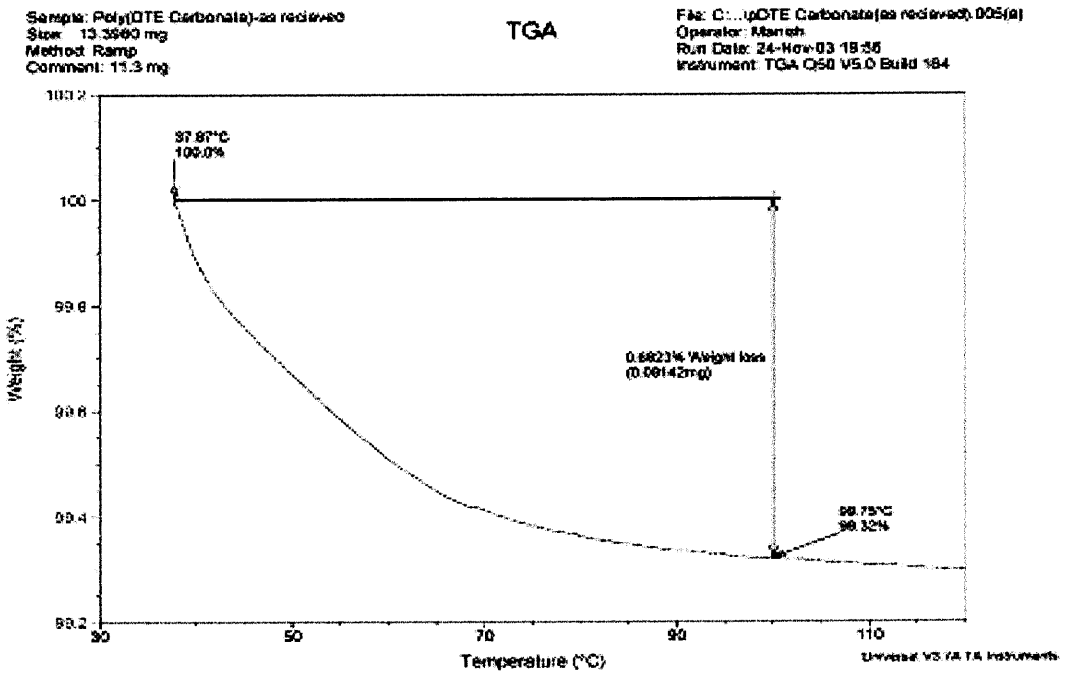

Figure C.3 TGA Result of poly(DTE) Carbonate as received.

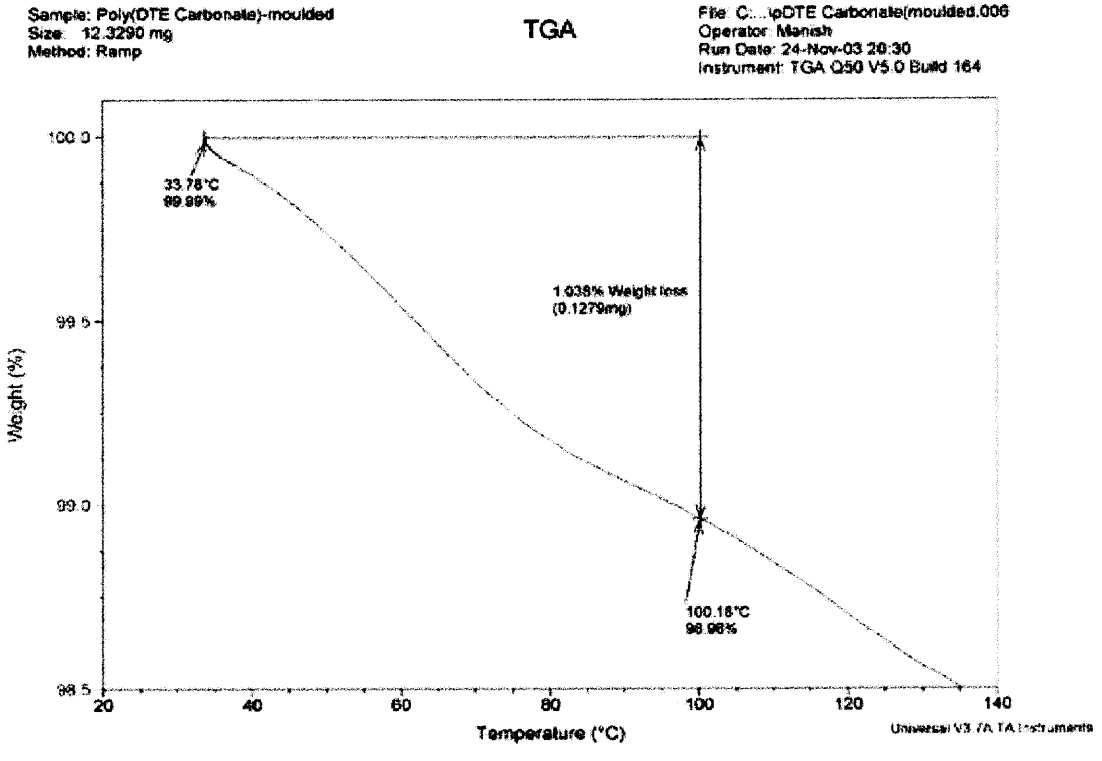

Figure C.4 TGA Result of poly(DTE) Carbonate in molded form.

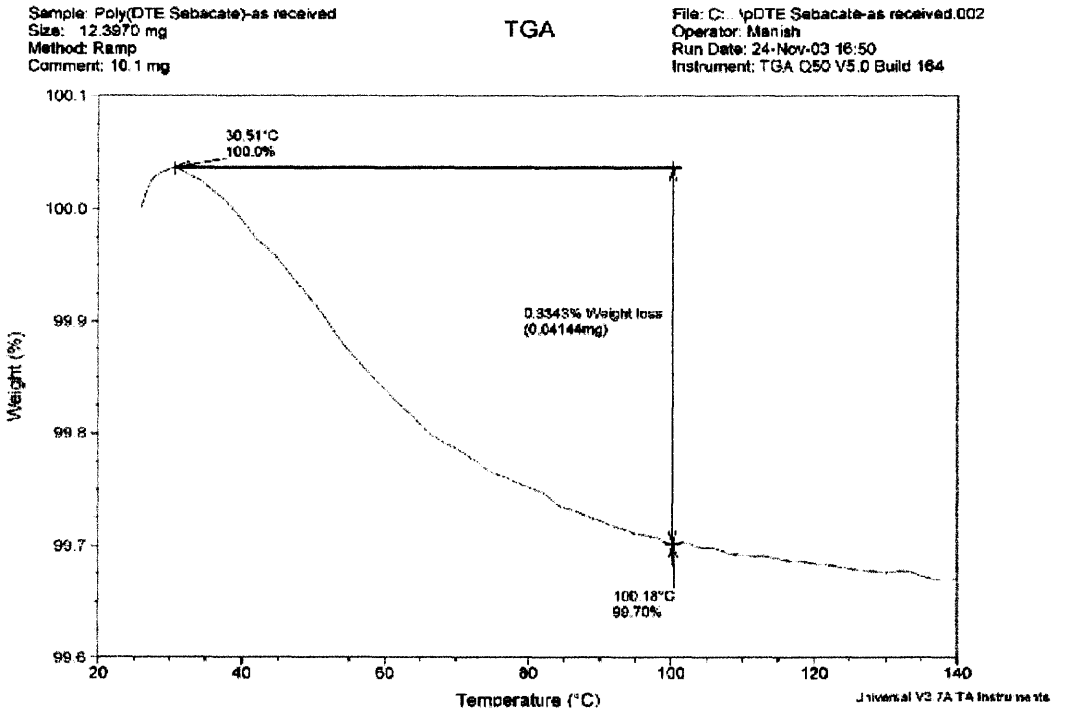

Figure C.5 TGA Result of poly(DTE) Sebacate as received.

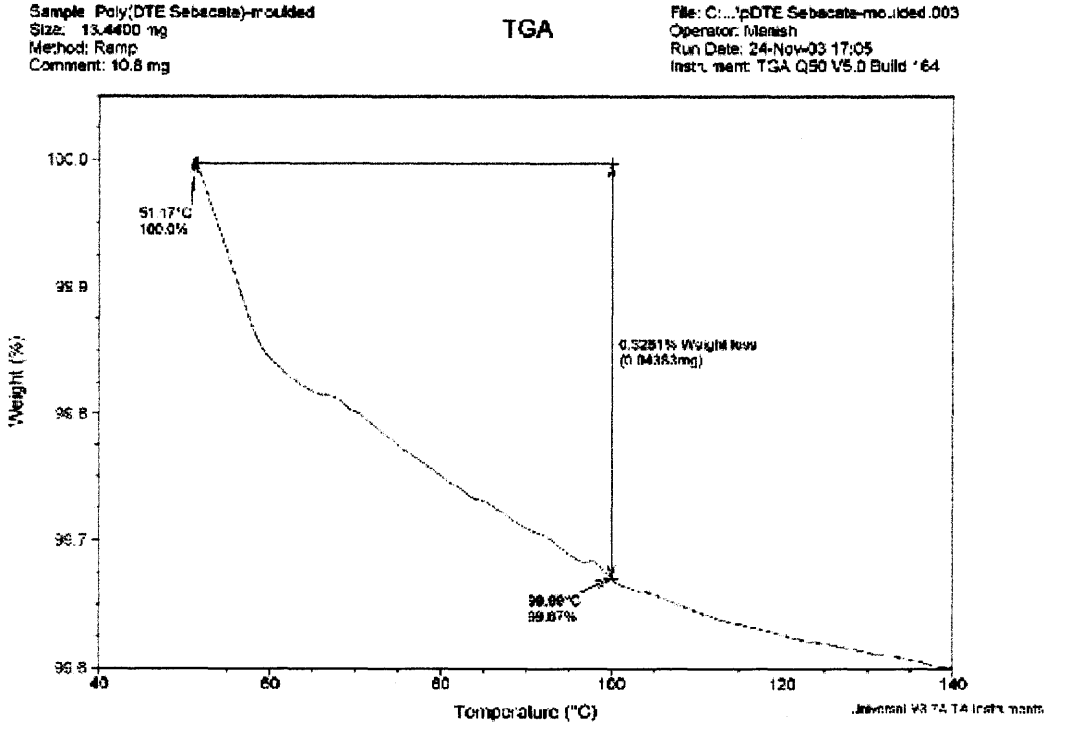

Figure C.6 TGA Result of poly(DTE) Sebacate in molded form.

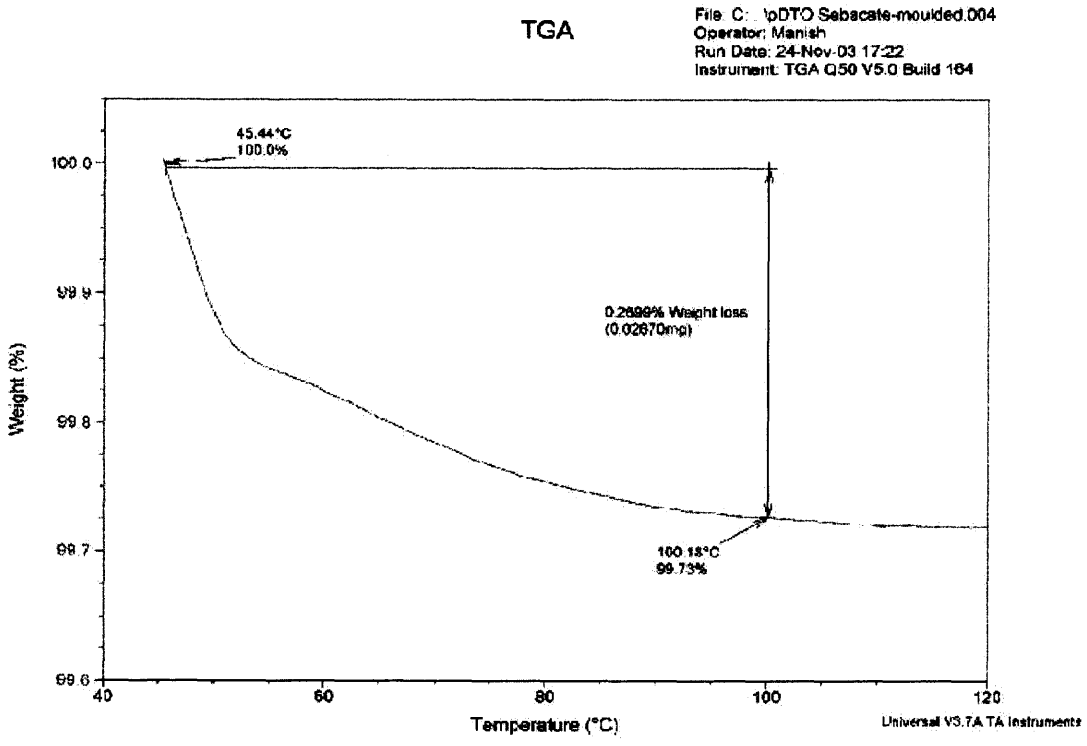

Figure C.7 TGA Result of poly (DTO) Sebacate in molded form.

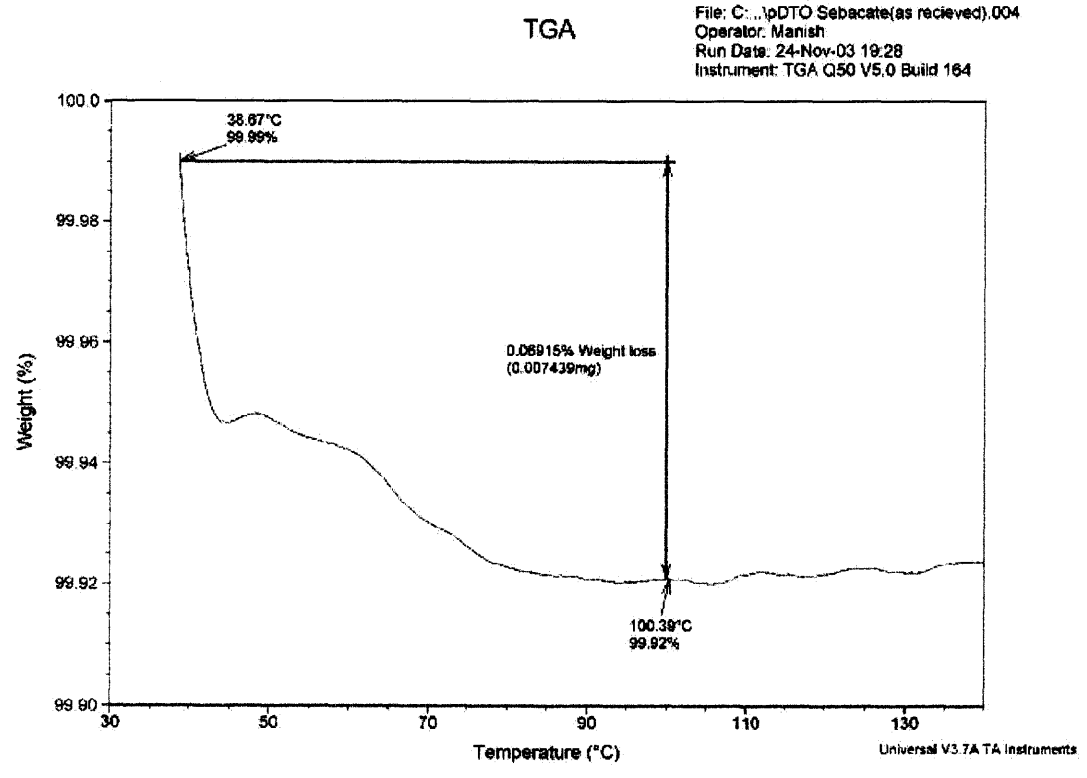

Figure C.8 TGA Result of poly(DTO) Sebacate as received.

APPENDIX E

DSC TEST RESULTS

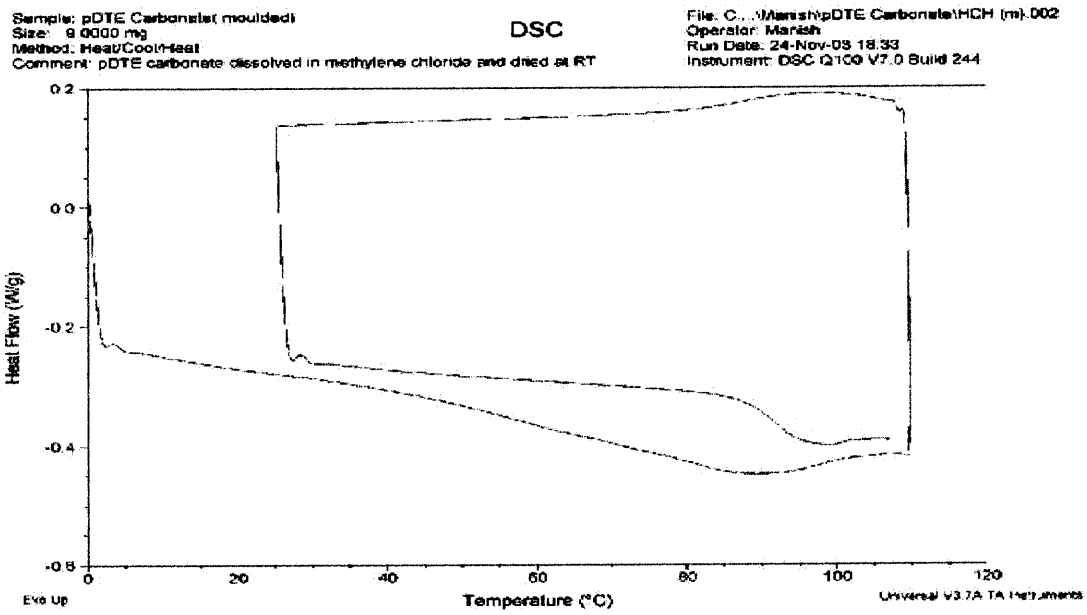

Figure E.1 DSC result of poly(DTE) Carbonate in molded form.

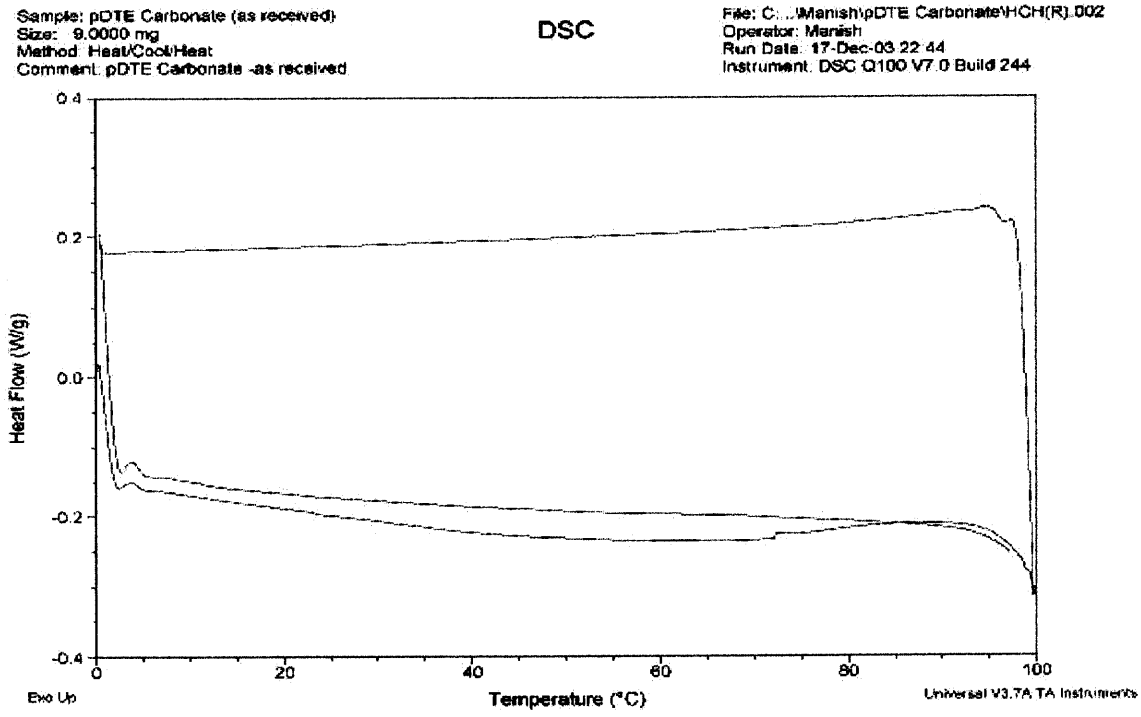

Figure E.2 DSC result of poly(DTE) Carbonate as received.

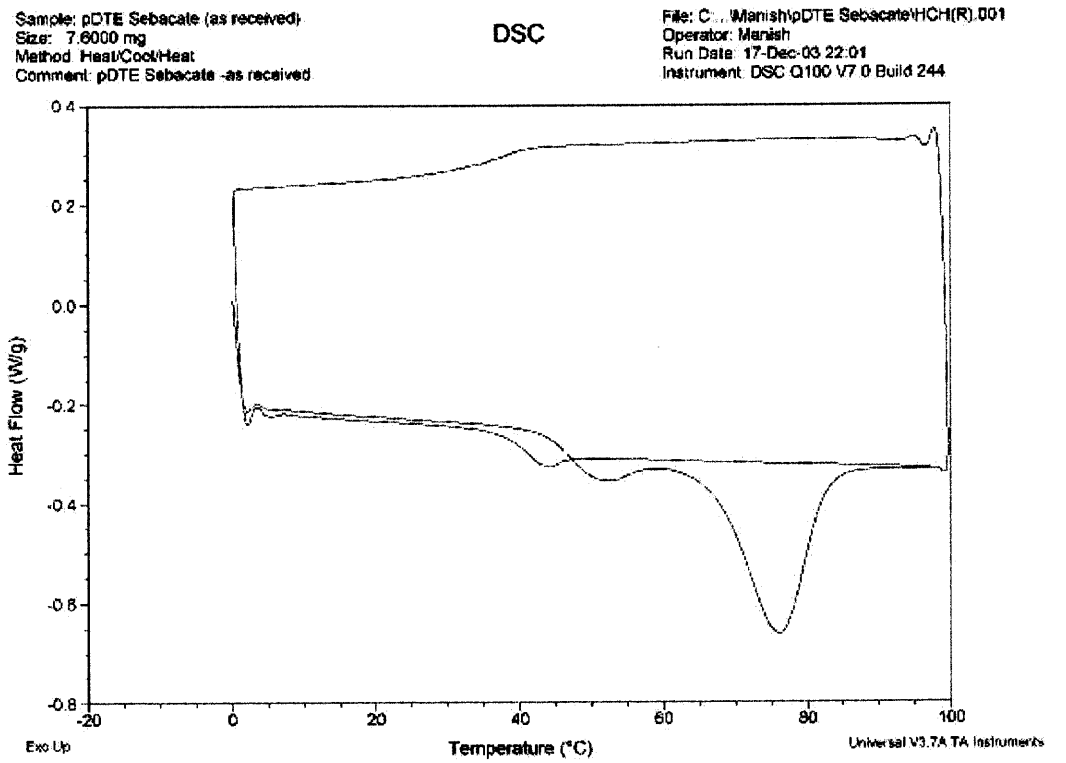

Figure E.3 DSC result of poly(DTE) Sebacate as received.

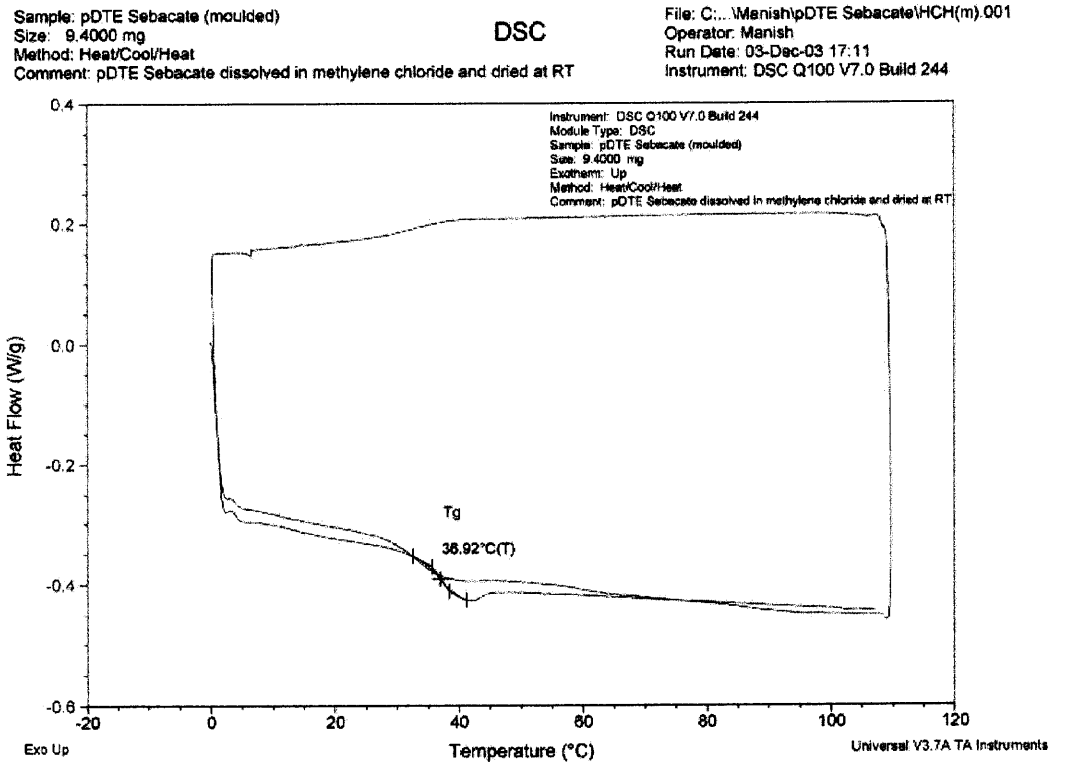

Figure E.4 DSC result of poly(DTE) Sebacate in molded form.

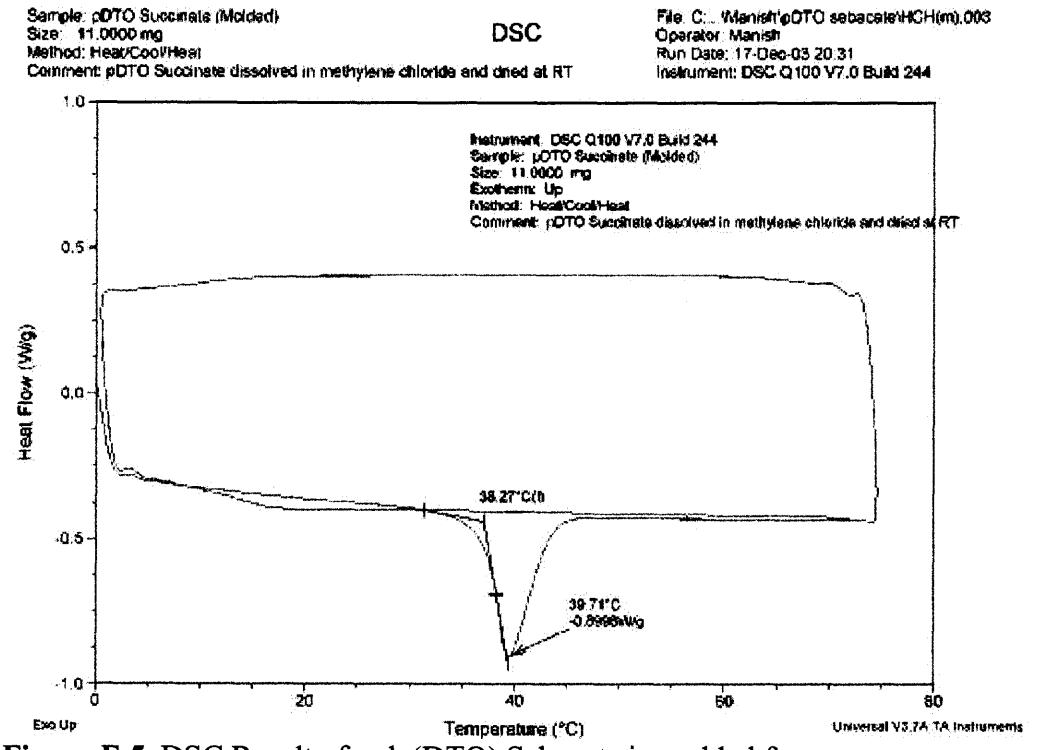

Figure E.5 DSC Result of poly(DTO) Sebacate in molded form.

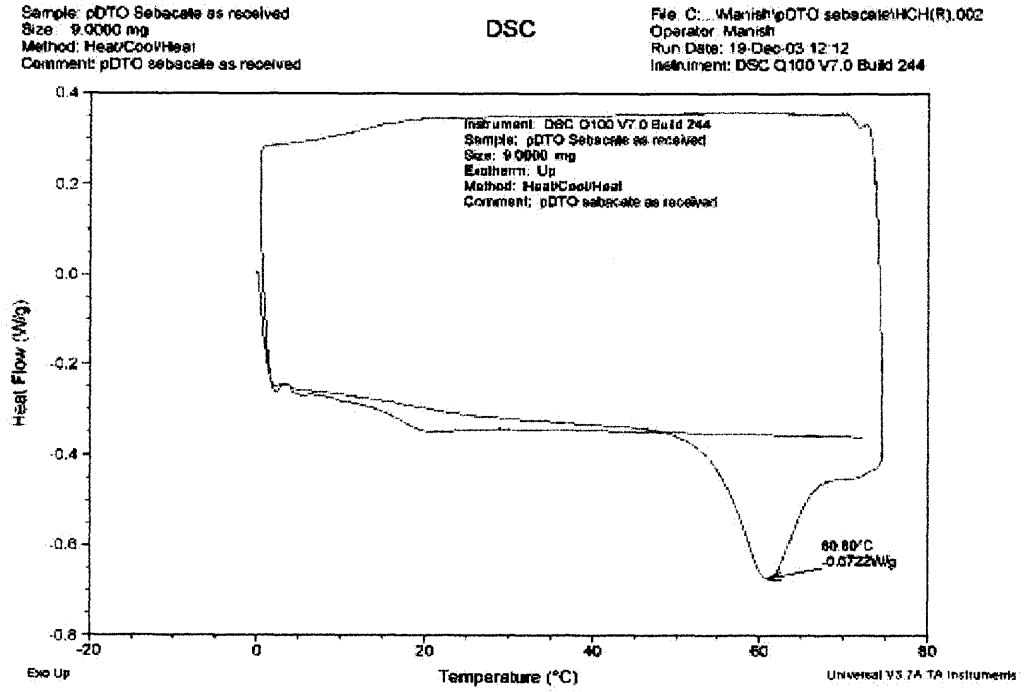

Figure E.6 DSC Result of poly(DTO) Sebacate as received.

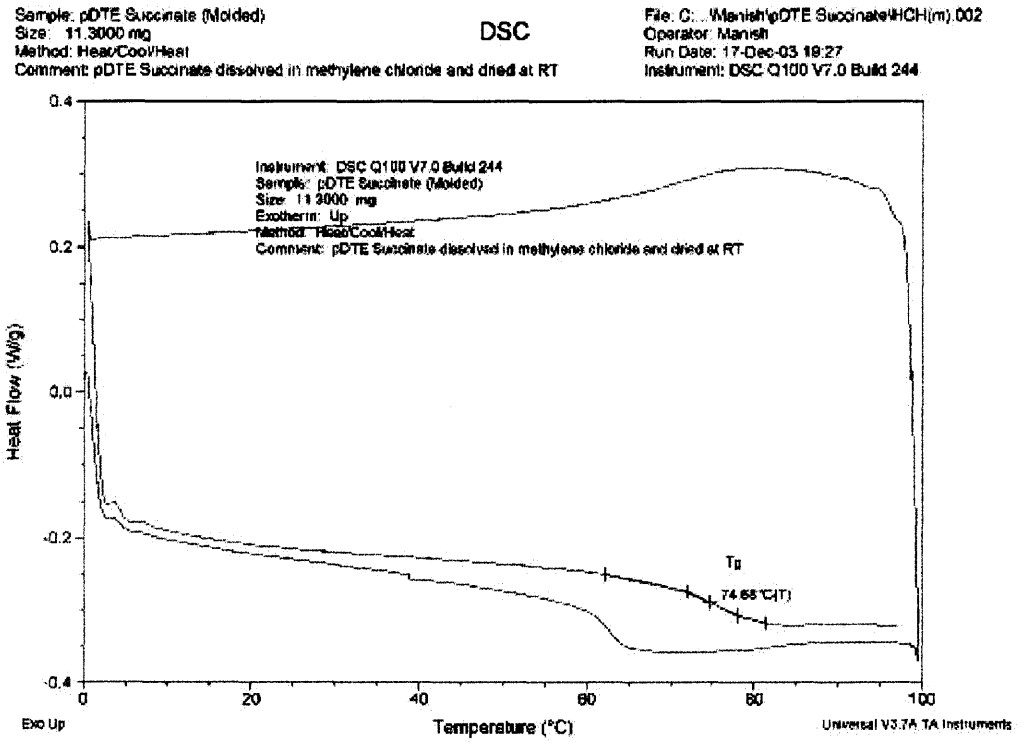

Figure E.7 DSC Result of poly(DTE) Succinate in molded form.

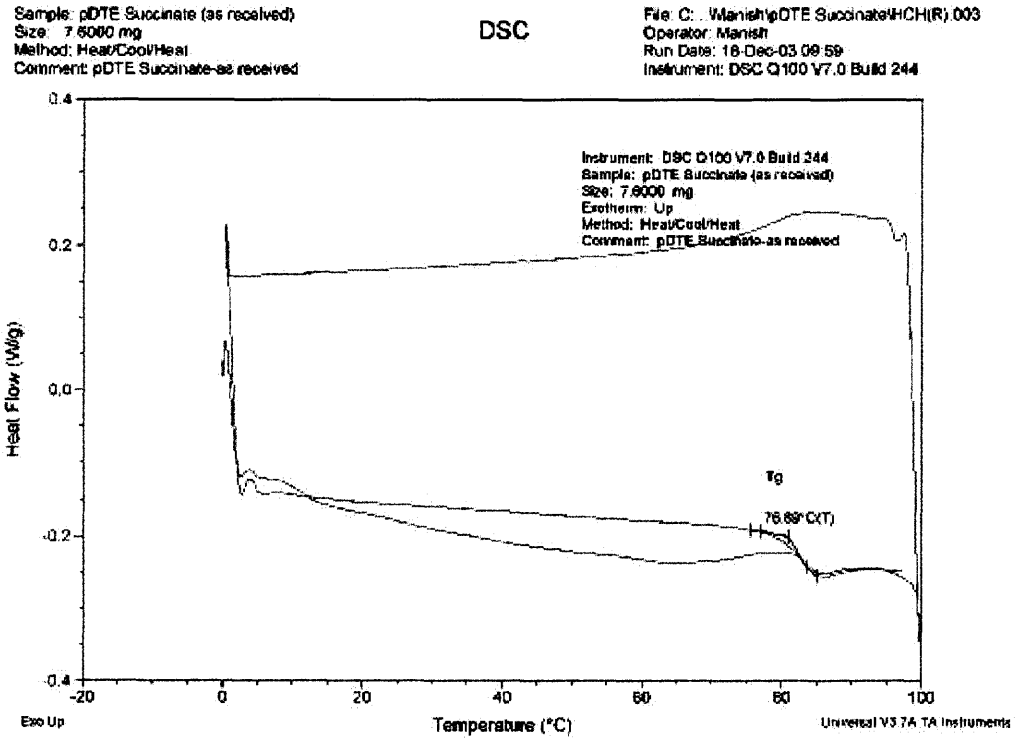

Figure E.8 DSC Result of poly(DTE) Succinate as received.

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